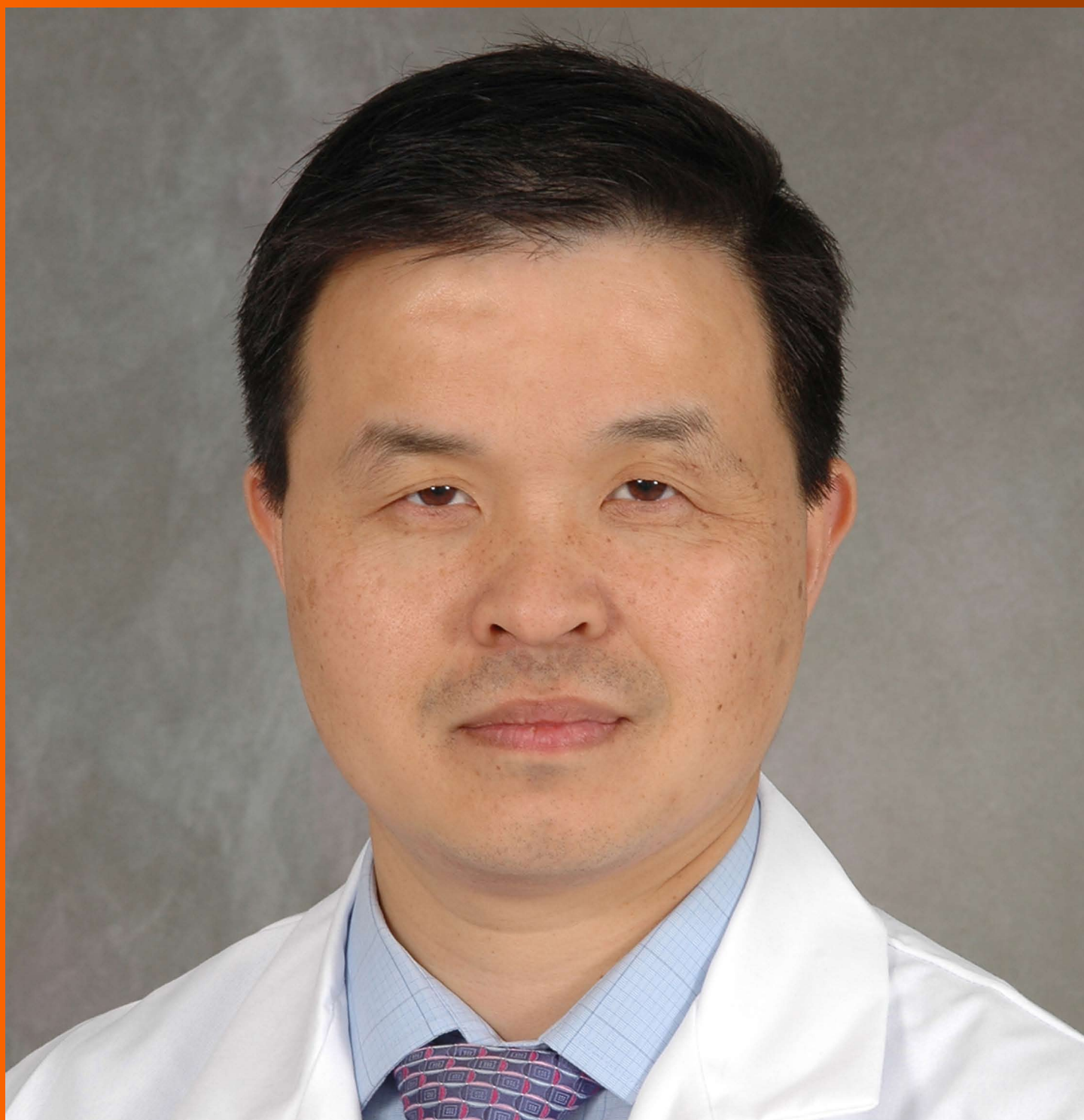


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Abnormal DNA methylation as a cell-free circulating DNA biomarker for colorectal cancer detection: A review of literature

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Abstract

Colorectal cancer (CRC) is one of the most prevalent malignancies in the world. CRC-associated morbidity and mortality is continuously increasing, in part due to a lack of early detection. The existing screening tools such as colonoscopy, are invasive and yet high cost, affecting the willingness of patients to participate in screening programs. In recent years, evidence is accumulating that the interaction of aberrant genetic and epigenetic modifications is the cornerstone for the CRC development and progression by alternating the function of tumor suppressor genes, DNA repair genes and oncogenes of colonic cells. Apart from the understanding of the underlying mechanism(s) of carcinogenesis, the aforementioned interaction has also allowed identification of clinical biomarkers, especially epigenetic, for the early detection and prognosis of cancer patients. One of the ways to detect these epigenetic biomarkers is the cell-free circulating DNA (circDNA), a blood-based cancer diagnostic test, mainly focusing in the molecular alterations found in tumor cells, such as DNA mutations and DNA methylation.

In this brief review, we epitomize the current knowledge on the research in circDNA biomarkers - mainly focusing on DNA methylation - as potential blood-based tests for early detection of colorectal cancer and the challenges for validation and globally implementation of this emergent technology.

Key words: Colorectal cancer early detection; Colorectal cancer screening; Circulating free DNA; Colorectal cancer blood-based biomarkers; DNA methylation blood biomarkers

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Core tip: Colorectal cancer (CRC) is one of the most prevalent malignancies in the world. CRC-associated morbidity and mortality is continuously increasing, in part due to a lack of early detection. The main aim of this article is the brief description of the basic screening modalities and their efficacy for CRC detection, the process of colorectal carcinogenesis and how the molecular pathways of CRC (focusing on epigenetic modifications) influence the clinical application of new blood-based biomarkers such as circDNA. Then we will focus on the most recent findings concerning the studies on circDNA, mainly related to DNA methylation and the challenges for validation and globally implementation of this emergent technology.

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INTRODUCTION

Colorectal cancer (CRC) is the most common malignancy presented in gastrointestinal (GI) tract and the third most frequent cancer globally, with an incidence approximately approaching 1.5 million cases per year^[1,2]. Likewise, it is considered that over 600000 deaths occur each year by neoplasms of the large bowel making them, the third commonest cause of cancer-related deaths^[2]. It is thought that the gradual adoption of Westernized lifestyle and dietary habits by the majority of the countries in association with aging of the population are responsible for the increase in morbidity and mortality rates from CRC. This is in accordance with the World Health Organization which estimates that a substantial increase in the number of newly diagnosed CRC cases and a 80% rise in deaths from CRC are expected by 2030^[3]. It should also be pointed out that colorectal adenocarcinomas are distinctive for their relatively fast progression and late clinical presentation, characteristics that are fairly preventable if identified at an early stage.

Nevertheless, the currently available screening tests for the early detection of CRC need improvement enough in order to increase their cost-effective status. Thus, it is conceivable that, there is a significant interest in using noninvasive blood biomarkers which could be of low cost and high sensitivity and specificity to help reduce the predicted surge in the incidence of CRC by identification and removal of a larger number of polyps that potentially could lead to CRC over time^[3]. These biomarkers are designed to detect molecular indicators in the plasma or serum such as DNA, RNA or protein in order to expand the existing list of CRC screening modalities^[4].

Epigenetic phenomena contribute to colorectal neoplasia^[5]. This term refers to the mechanisms that alter gene expression without changing their DNA sequence. Epigenetic phenomena may include DNA methylation, histone modification and chromatin regulation through non-coding RNAs (microRNAs, lncRNAs, etc.)^[6]. Since DNA methylation and DNA mutations detected in tumor cells, it is reasonable to assume that these alterations are reflected in circDNA released from neoplastic tissue into blood circulation. Testing for circDNA in the peripheral blood could serve as an important candidate biomarker for the detection of CRC at early stages. An existing paradigm of commercial blood test for CRC detection in the circDNA is the monitoring of methylation of the septin 9 gene (*SEPT9*) promoter region.

Therefore, the aim of this article is the brief description of the basic screening modalities and their efficacy for CRC detection, the process of colorectal carcinogenesis and how the molecular pathways of CRC (focusing on epigenetic modifications) influence the clinical application of new blood-based biomarkers such as circDNA. Then we will focus on the most recent findings concerning the studies on circDNA, mainly related to DNA methylation and the challenges for validation and globally implementation of this emergent technology.

EXISTING SCREENING MODALITIES TO CRC

Screening modalities

There are various strategies for screening nowadays; the most accepted being the colonoscopy, and the combination of sigmoidoscopy and fecal occult blood test (FOBT). The high sensitivity and specificity has established the colonoscopy the cornerstone for the early identification of colonic malignancies in the average-risk population^[7,8]. There are some drawbacks that limit the desired wide acceptance. As an invasive examination, complications may be unavoidable, the most common being cardiovascular events during the procedure and the post-polypectomy bleeding and perforation^[7]. Other disadvantages could be a significant miss rate of lesions even for large colonic abnormalities, its high cost and the low acceptance level by the population^[9].

Compared to colonoscopy, sigmoidoscopy has quite few disadvantages due to low cost, less preparation time

and no need for sedation^[10]. The main problem is the ability to detect only the lesions of distal colon making the decision of performing colonoscopy a subject for controversy even to date.

The third and most frequently applied screening test is FOBT^[11]. Although these tests are easier to perform than colonoscopy or sigmoidoscopy, they are associated with false positive and false negative results due to diet, other conditions like colitis and hemorrhoids and the effect of temperature on the samples^[12]. Moreover, FOBT cannot be used as solo screening test, as a positive results lead to colonoscopy performance^[13].

Thus, there is an emergent need for new screening tests such as blood-based test which could detect CRC earlier, increase patient participation with minimal risks, costs, and false positive and negative results.

MOLECULAR PATHWAYS IN CRC AND DNA METHYLATION

CRC molecular pathways

Colorectal cancer is a multifarious disease. The comprehension of the molecular pathways involved in its development, will help to optimize the screening procedure based on distinctive pathologic and molecular features of the malignancies. Three basic pathways of colorectal carcinogenesis have been recognized since 1990 that is, Chromosomal Instability (CIN), Microsatellite Instability (MSI) and CpG island methylator phenotype (CIMP) pathway^[14].

Chromosomal instability, also entitled "the suppressor pathway", was first introduced in 1990 by Fearon *et al.*^[14] and is the most frequent etiology for gene alteration in colorectal neoplasia. Its main characteristic is the modification of whole chromosome or some of its regions, affecting important genes leading to carcinogenesis. These genomic defects provoke suppressor genes inactivation as Deleted in Colon Carcinoma (*DCC*), SMAD family member 2 (*SMAD2*), SMAD family member 4 (*SMAD4*), Adenomatosis polyposis coli (*APC*) and tumor protein p53 (*TP53*) and oncogene activation such as the human homolog of the Kirsten rat sarcoma-2 virus oncogene (*KRAS*)^[15]. The accumulation of these modifications seems to play the most crucial role for cancer to develop and not the sequence of their presentation as once considered. The second model which involved in normal intestinal mucosa transformation to malignancy is the microsatellite instability. MSI is another type of genomic instability which refers to deletions or insertions of a few nucleotides in genes responsible for repair during DNA replication, the DNA mismatch repair (MMR) genes^[16]. This aberrant genomic region mainly segregates in repetitive DNA nucleotide unit (microsatellites) throughout the genome resulting in the inactivation of MMR genes (*i.e.*, *MSH2*, *MLH1*, *MSH6*, *PMS1-2*, *MLH3*, *MSH3*, *ExoI*). It is well-known that this route of carcinogenesis is involved in Lynch syndrome and for a notable proportion of sporadic CRC (15%-20%)^[17]. The third model involved in the

CRC development and progression, is CIMP which refers to the presence of simultaneous hypermethylation of multiple genes. It belongs to the epigenetic mechanisms leading to silence gene function after methylation at the 5'-CG-3'(CpG) dinucleotide in the promoter region of many genes (*APC*, *MCC*, *MLH1*, *MGMT*), resulting therefore, in inactivation of tumor suppressor genes^[18]. CIMP is accountable for 15%-20% of sporadic CRC and according to the study of Jass^[19], we are able to classify CRC according to the presence of MSI and CIMP as Figure 1 shows.

DNA methylation and its role in CRC carcinogenesis

DNA methylation mainly occurs in specific parts of the genome called, as we have seen previously, CpG islands. Considering the stability of DNA methylation compared to mutations, we may presume that methylation is a favorable area for biomarker exploration.

The concept that genome methylation may play a critical role in specific steps in the CRC carcinogenesis has been expressed in 1983 by Feinberg and Vogelstein^[20] who showed that at early stages of CRC there is a DNA hypomethylation, mainly located at CpG islands. They also demonstrated that this loss of methylation was combined by hypermethylation and inactivation of tumor suppressor or DNA repair genes^[21]. This epigenetic modification has recently been associated with the normal mucosa-aberrant crypt focus (ACF)-adenoma-carcinoma sequence, playing an important role in CRC development^[22]. Consequently, DNA methylation appears to be one of the cornerstones of carcinogenesis because it occurs at the first steps of CRC process; involves CIMP pathway with MSI, as hypermethylation of MMR genes results in MSI sporadic CRC; through CIMP, it has been linked with CIN in colon malignancy (promoter methylation of *GATA4*, *GATA5*, *p16* resulted in chromosomal loss or gain); and finally it is implicated in each of these paths through many abnormally methylated genes as recently studies have revealed^[23-28] (Figure 2). The design of genetic and epigenetic biomarkers, especially those related to detection of aberrant methylated genes able to offer the maximum coverage of intestinal neoplasia, seems to be a reasonable approach. Accordingly, several studies have been performed the last decade, for the potential use of DNA markers in different biologic fluids as strategies for colorectal carcinoma early detection^[29].

CELL-FREE CIRCULATING DNA AS A BLOOD-BASED BIOMARKER

Mandel and Metais^[30] in 1948, were the pioneers who discovered the existence of cell-free nucleic acids (cfNAs) in blood, leading to "discrimination" of affected patients from healthy controls, even though the first reported presence of cfNAs was in 1869^[31]. Since then, several studies have been made, especially related to cancer pathogenesis, showing that malignant-nucleic acids could be present in different "body fluids" (*i.e.*,

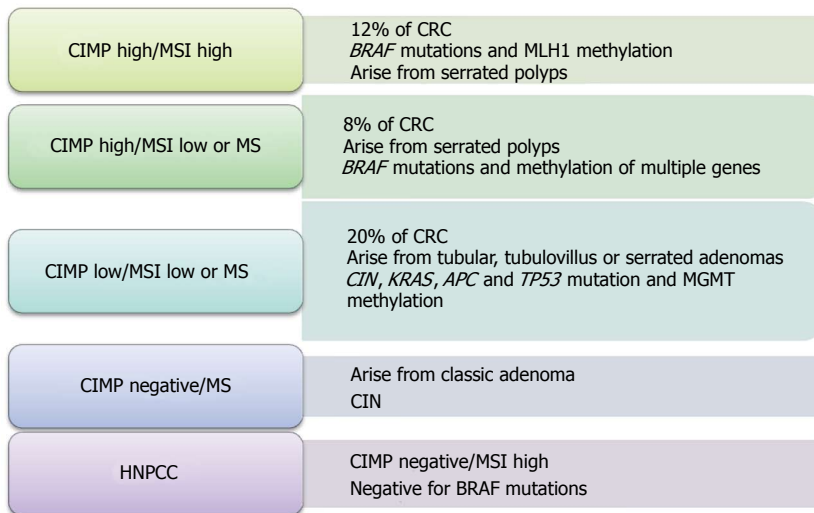


Figure 1 Molecular classification of colorectal cancer. The figure shows the different molecular profile and clinic-histopathological characteristics of each classification. CIMP: CpG island methylator phenotype; MSI: Microsatellite instability; MS: Microsatellite stability; CRC: Colorectal cancer; CIN: Chromosomal instability; HNPCC: Hereditary nonpolyposis colorectal cancer; MGMT: O-6-methylguanine-DNA methyltransferase; BRAF: v-raf murine sarcoma viral oncogene homolog B1; MLH1: MutL homolog 1; KRAS: Kirsten rat sarcoma 2 viral oncogene homolog; APC: Adenomatosis polyposis coli; TP53: Tumor protein p53.

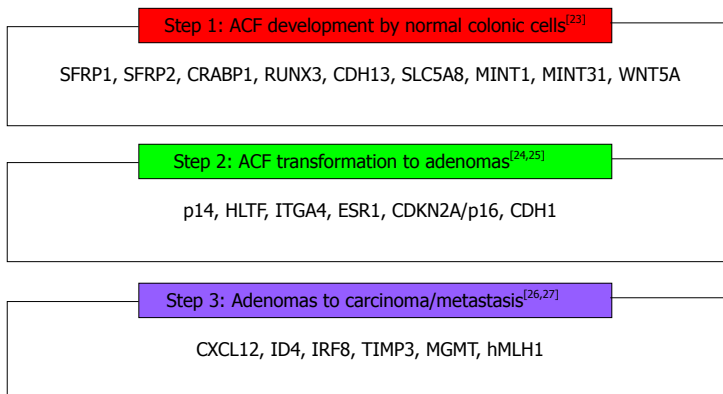


Figure 2 The figure exhibits the most frequent methylated genes/loci involved in step-by-step adenoma-carcinoma process in the context of colorectal cancer development. SFRP1: Secreted frizzled-related protein 1; CRABP1: Cellular retinoic acid binding protein 2; RUNX3: Runt-related transcription factor 3; CDH13: Cadherin 13; SLC5A8: Sodium solute symporter family 5 member 8; MINT1: Methylated in tumor locus 1; WNT5A: Wingless-type MMTV integration site family, member 5A; p14: Tumor protein 14; HLF: Helicase-like transcription factor; ITGA4: Integrin, alpha 4; ESR1: Estrogen receptor 1; CDKN2A/p16: Cyclin-dependent kinase inhibitor 2A; CDH1: E-cadherin; CXCL12: Chemokine (C-X-C) ligand 12; ID4: Inhibitor of DNA binding 4; IRF8: Interferon regulatory factor 8; TIMP3: Tissue inhibitor of metalloproteinase 3; MGMT: O-6-methylguanine-DNA methyltransferase; hMLH1: MutL homolog 1.

stool, blood, urine). Therefore, it was a matter of time the establishment of the potential advantages using cfNA as noninvasive neoplasia detection^[32,33]. The first report referring to detection of “abnormal” cfNAs in CRC patients took place in 1992, when Vogelstein *et al.*^[34] discovered *KRAS* gene mutation in stools samples of CRC-affected individuals. Since then, a great number of studies have performed evaluating other than *KRAS*, genome modifications directly expressed by cfNAs. These modifications are characterized mainly by analysis of high mutation frequency genes (*KRAS*, *TP53*, and *APC*), MSI, Loss of Heterozygosity (LOH), DNA, and microRNA methylation changes. Except the latter one, all the others are key factors for colorectal carcinogenesis which could be expressed by increased circDNA concentrations in blood of CRC patients compared with healthy controls, first mentioned by Leon *et al.*^[35] in 1977, followed by many other studies^[35-37].

Thus, since circDNA in blood reflects significant genome alterations emerging during CRC carcinogenesis, it could be used systematically as a potential biomarker for early detection of colonic malignant tumors, especially after the recent advances in next generation sequencing (NGS) technology^[38]. Here follows a discussion of methods to detect circDNA-based markers in blood and the studies focusing on description of these markers namely, aberrant DNA methylation and mutations, microsatellite

alterations, DNA modifications in mitochondria, integrity and quantification of DNA.

Methods to detect methylation-related circDNA markers in blood

Methodologies suitable to investigate and detect in serum or plasma frequently methylated genomic regions, offering high detection rate is more than an appealing aim. These ideal markers should have low levels of background methylation so as to avoid the decreased specificity and increased false-positive results. Several molecular approaches are currently performed to identify circDNA in blood. Conventional methylation-specific PCR, though very sensitive method, presents high levels of false positive and negatives results and its qualitative method to interpret the findings, limits its clinical utility^[39]. On the other hand, methods based on quantitative methylation-specific PCR (*i.e.*, MethyLight, SMART-methylation-specific PCR) offers the opportunity to select more easily the methylation thresholds and avoid false positive results identifying incomplete bisulfite conversion^[40]. Other approaches include Methylation Array, DNA array, Surface-Enhanced Raman Scattering (SERS), and Restriction Fragment Length Polymorphism (RFLP). Another interesting and “more digital” methodology, called “Methyl-BEAMing”, was demonstrated by Li *et al.*^[41] in order to digitally quantify cancer-derived vimentin DNA, confronting the issues with

Table 1 DNA integrity index in colorectal cancer patients

Ref.	Increased DNA integrity	Decreased DNA integrity
Umetani <i>et al</i> ^[45] , 2006	Yes	-
Da Silva Filho <i>et al</i> ^[46] , 2013	Yes	-
Leszinski <i>et al</i> ^[47] , 2014	Yes	-
Mouliere <i>et al</i> ^[48] , 2011	-	Yes
Mead <i>et al</i> ^[49] , 2011	-	Yes
Mouliere <i>et al</i> ^[50] , 2014	-	Yes
Yörüker <i>et al</i> ^[51] , 2015	-	Yes

the small fraction of blood circDNA.

Given the great concerns arouse by previous approaches concerning the inability for reproducibility and high sensitivity, promising results revealed by the study of Leary *et al*^[42]. The use of NGS approach showed encouraging results, distinguishing CRC patients at advanced stage from healthy controls. Thus, NGS could provide high sensitivity, covering large regions of genome for CRC early detection.

Cell-free circulating DNA-based markers

During the last two decades, circDNA has become a potential biomarker for diagnosis of malignant tumors, exhibiting their genetic and epigenetic modifications. It exists in the plasma or serum, being source of apoptotic, necrotic cancer cells or even living cells. It can appear as unbound DNA molecule; as histone part in nucleosome; or as portion of apoptotic cells. As already mentioned, there are several methods of assessing circDNA as a potential biomarker for detection of CRC at early stages. Herein, we would try to summarize the main characteristics of each method, noting presentative studies reflecting the potential clinical use of these circDNA-based modalities.

One of these methods is the quantification of circDNA levels in blood, studied thoroughly in CRC patients since the research by Leon *et al*^[35] in 1977 who proved that concentration of circDNA was higher than that of healthy persons. Additional studies, such as the ones performed by Frattini, Schwarzenbach *et al*^[43] respectively, verified the elevated circDNA levels in the plasma of CRC individuals compared with the non-cancerous controls^[43]. Although, patients with malignancies may present greater levels of circDNA than normal persons, it should be emphasized that circDNA in plasma may also be observed in other clinical entities like trauma, inflammatory disorders even in healthy individuals^[35].

In the recent years, it is well-established that the manner, with which the circDNA is released in blood-stream, reflects its size and morphology. The exact mechanism is yet to be clarified but it is believed that circDNA entered the blood by apoptosis and then it is fragmented by the action of nucleases or phagocytes, into small particles of 185 to 200 bp in length^[44]. The measure of the ratio of long circDNA fragments to short ones mirrors circDNA integrity. A great number of studies

have been performed, demonstrating inconsequent results (Table 1)^[45-51] as concerns the sensitivity and specificity of circDNA integrity index for CRC early detection. Interestingly, a recent research by Hao *et al*^[52] showed that the combination of DNA integrity index (ALU247/115 and ALU115 index) and carcinoembryonic antigen (CEA) detection may be efficient and reproducible method for early diagnosis of CRC. Therefore, larger clinical studies should be performed in order to limit the inconsistencies that circDNA integrity method exhibits.

Microsatellite alterations is another investigation field related to tumorigenesis of CRC and consist of MSI and LOH. Due to their presence in circDNA, it is assumed that they could be potential CRC biomarkers for early diagnosis of affected individuals. As we already have mentioned, *MSI* refers to deletions or insertions of a few nucleotides (1-6 bp in length) in genes responsible for repair during DNA replication, the MMR genes^[16], while *LOH* analysis emphasizes the loss of chromosomal parts carrying tumor suppressor genes. These somatic alterations have been detected in blood, nearly in 35% of all CRC patients. The existence of MSI-related circDNA fragments is known since the ending of 20th century followed by many studies focusing on the presence of MSI and LOH in circDNA^[53]. One of these by Hibi *et al* showed in 1999 that, although *LOH* and *MSI* found in 80% CRC patients when examined their microsatellite alterations, these shifts weren't verified upon the corresponding serum-based circDNA. Therefore, the available data reveals the relatively low sensitivity and specificity in diagnosing CRC at early stages when microsatellite alterations are investigated.

Similar disappointing results have been arisen from the study of circulating mitochondrial DNA (mtDNA) as potential circDNA-based biomarker for premature diagnosis of colonic neoplasia. Mitochondria are the cornerstone in energy metabolism, aging, and apoptosis, playing a crucial role in shifting the cell from scheduled death to abnormal cell growth, thus having potential contribution to the carcinogenesis^[54]. An important part of mtDNA is its D-loop region, a noncoding region which involves the expression and organization of the mitochondrial genome. It is hypothesized that this part of mtDNA is a hotspot of mutations leading to DNA instability, opinion that has been verified in several types of cancers such as, head and neck, colorectal, stomach, prostate, breast^[55]. Despite the initial encouraging signs, mtDNA shows reduced detection rate of early stage CRC, as the study by Hibi *et al*^[56] revealed, where the discovery of mtDNA modifications (somatic mutation in D-loop region) in tissues of early CRC patients haven't been noted in their circDNA.

As it is stated previously, the blood-based circDNA in CRC patients is composed by important molecules which have implicated in tumorigenesis process. Since 1992 when Vogelstein *et al*^[34] discovered *KRAS* gene mutation in stools samples of CRC-affected individuals, high mutation frequency genes, as *KRAS*, *TP53*, and *APC*

have been used as potential markers in circDNA analysis for early diagnosis of colonic malignant lesions^[57,58]. The results were discouraging due to low concentration of tumor circDNA (based on the somatic mutations analysis of *KRAS/TP53/APC* genes modification) in CRC patients compared with the wild-type circDNA in non-CRC individuals^[58]. Moreover, it should be noted that even the use of NGS circDNA detecting method, hasn't offered any improvement in detection of these aberrant tumor DNA mutations^[59]. Finally, as aforementioned, genes such as *APC*, *TP53*, and *KRAS* are mutated in a great degree of CRC cases, spreading over different parts of genome, making mutational assessment difficult. Thus, it is reasonable to assume that very large genomic regions would need to be evaluated in order to obtain a respectable sensitivity and in combination of the unique presentation of modified genes in each patient, it is still difficult enough to use somatic mutations for CRC early detection.

Abnormal DNA methylation as a cell-free circulating DNA biomarker

As it has been already highlighted, the critical role of abnormal DNA methylation to specific steps in the CRC carcinogenesis has been expressed since 1983 from Feinberg and Vogelstein^[20]. Since then and during the recent years, many studies have revealed that this epigenetic modification has been associated with the normal mucosa-ACF-adenoma-carcinoma sequence, playing an important role in CRC development, mainly, at early stages^[22-27]. It is known that during DNA methylation, DNA methyl transferases (DNMTs) catalyze the addition of a methyl group (-CH₃) to the fifth carbon position on cytosines within CpG dinucleotides. The latter, although spread over throughout the human genome, they are frequently discovered in the promoter regions of nearly 70% of genes, usually named as "CpG-islands"^[60]. Furthermore, it is well-established by now that hypermethylation of tumor suppressor promoters genes could induce transcriptional gene silencing, resulting on aberrant cellular signaling and therefore potential initiation of tumorigenesis process^[61]. Moreover, it is interesting that methylation could happen in CpG sites throughout the genomic body and not necessarily only in promoter regions leading though to transcriptional activation^[62]. On the other hand, global hypomethylation which frequently presented prematurely during carcinogenesis, exhibits loss of DNA methylation throughout the genome, resulting on CIN and cell mutation^[63]. Consequently, the significance of aberrant DNA methylation led to investigation and discovery of blood-based mainly, due to its noninvasiveness and cost-effectiveness, CRC detection biomarkers.

One of the most investigated genes is the *SEPT9* gene involved in cellular proliferation control. The methylation of v2 promoter region of *SEPT9* has been demonstrated in CRC biopsy lesions compared with normal tissues. According to Grützmann *et al.*^[64], its detection in plasma of CRC patients

exhibited a sensitivity of 72% and specificity of 90%, something that was validated by the study of Warren *et al.*^[65]. Nevertheless, a recent prospective trial performed by Church *et al.*^[66] investigated the *SEPT9* methylation in 7941 asymptomatic individuals during screening with available assay showing a CRC detection rate up to 48.2% and specificity up to 91.5%. Obviously, the need of further researches upon this commercially available test is indispensable not only to improve its detection rate but also to discover new assays for *SEPT9* methylation detection. Furthermore, researchers understanding the usefulness of *SEPT9* have assessed potential combinations with other methylation biomarkers. Tänzer *et al.*^[67] have shown that methylated DNA from advanced premalignant intestinal lesions could be discovered using the panel of aristaless-like homeobox 4 (*ALX4*), and *SEPT9* markers. Similarly, Kostin *et al.*^[68] compared the methylation status of *SEPT9*, Helicase-like transcription factor (*HLTF*) and *ALX4* genes in macroscopically findings compatible with colorectal cancer ($n = 55$) and morphologically intact areas of the large bowel ($n = 71$), showing that this panel of biomarkers characterized by a sensitivity nearly to 74%-88% and a specificity 90%-96% for CRC early identification. Finally, He *et al.*^[69] demonstrated high sensitivities (81%-84%) and specificities (87%-90%) for noninvasive blood-based testing for initial-phase CRC, using multiplex MethyLight PCR assay to detect concomitantly, aberrant methylation pattern of *ALX4*, *SEPT9*, or transmembrane protein with EGF-like, and two follistatin-like domains 2 (*TMEFF2*) genes.

Apart from the aforementioned *SEPT9*-combined panels, there are recently studies showing even greater CRC detection rates if combined analysis of several genes is used. Alhquist *et al.*^[70] presented high overall sensitivity (87%) for the CRC detection compared with *SEPT9* (60%), using the combination of methylated genes such as bone morphogenetic protein (BMP3), N-myc downstream regulated family member 4 (NDRG4), vimentin, tissue factor pathway inhibitor-2 (TFPI2), mutant *KRAS* and β -actin. According to Carmona *et al.*^[71], there is a 78% sensitivity for CRC early diagnosis when combining angiotensin II receptor type 1 (*AGTR1*), wingless-type MMTV integration site family member 2 (*WNT2*), slit homolog 2 (*Slit2*) (*SLIT2*) genes. Moreover, Cassinotti *et al.*^[72] exhibited the potential use of gene panel, consisting of D-type cyclin gene (*CYCD2*), hypermethylated in Cancer 1 (*HIC1*), *PAX5*, Ras association domain family 1, isoform A (*RASSF1A*), retinoblastoma tumor suppressor (*RB1*) and sheep red blood cells (SRBC) with sensitivity nearly 84% and specificity 68%. Comparable results revealed by others studies making these panels powerful tools for future large-scale trials^[73-95] (Table 2).

In parallel, several blood-based methylated genes as potential biomarkers have been studied either alone or within panels as previously demonstrated, and a summary of them exhibited in Table 2, concerning their detection rate^[41,64-67,70,72,74-92]. Some of them (*SEPT9*, *ALX4*, *SDC2*, *RUNX3*, *TMEFF2*, *NEUROG1*) present high sensitivity and

Table 2 Abnormally methylated genes as potential circDNA blood-based colorectal cancer detection biomarkers

Potential biomarkers	CRC sensitivity (%)	CRC specificity (%)	Ref.
ALX4	40-83	70-82	[67,76]
TFPI2	76-89	-	[77-80]
SDC2	92	-	[81]
RUNX3	65	100	[82,83]
NEUROG1	52-64	91	[84]
MGMT	39	96	[74]
RARβ2	24	100	[74]
NGFR	51	84	[85]
9-Sep	48-90	86-93	[64-67,70,86]
TMEFF2	65	69	[85]
Vimentin	59	93	[41]
RASSF2A	58	100	[74]
Wif-1	74	98	[74]
APC	6	100	[87]
hMLH1	43	98	[87]
HTLF	21-34	98-100	[87,88]
SFRP2	67	94	[89]
CDKN2A/P16	71	100	[83]
Panel: SEPT9, HTLF and ALX4	74-88	90-96	[68]
Panel: SEPT9 and ALX4	-	-	[67]
Panel: MGMT, RASSF2A, Wif-1 gene	86.5	-	[74]
Panel: BMP3, NDRG4, vimentin, TFPI2, mutant KRAS and β-actin	87	-	[69]
Panel: AGTR1, WNT2, SLIT2	78	-	[71]
Panel: CINP1, FBN1, INA, SNCA, MAL and SPG20	90-99	-	[73]
Panel: CYCD2, HIC1, PAX5, RASSF1A, RB1 and SRBC	84	68	[72]
Panel: THBD and C9orf50	71	80	[75]
RASSF1A, E-cadherin	-	-	[72,90]
CAHM	-	-	[91]
FRP2, TPEF/HPP1	-	-	[83,84,92]

ALX4: Aristaless-like homeobox 4; TFPI2: Tissue factor pathway inhibitor 2; SDC2: Syndecan 2; RUNX3: Runt-related transcription factor 3; NEUROG1: Neurogenin 1; MGMT: O-6-methylguanine-DNA methyltransferase; RARβ2: Retinoic acid receptor β2; NGFR: Nerve growth factor receptor; SEPT9: Septin 9; TMEFF2: Transmembrane protein with EGF-like, and two follistatin-like domains 2; RASSF2A: Ras association domain family 2 (isoform A); Wif-1: Wnt inhibitory factor-1; APC: Adenomatosis polyposis coli; hMLH1: MutL homolog 1; HTLF: Helicase-like transcription factor; SFRP2: Secreted frizzled-related protein 2; CDKN2A/P16: Cyclin-dependent kinase inhibitor 2A; BMP3: Bone morphogenetic protein; NDRG4: N-myc downstream regulated family member 4; KRAS: Kirsten rat sarcoma 2 viral oncogene homolog; AGTR1: Tissue fac angiotensin II receptor type 1; WNT2: Wingless-type MMTV integration site family member 2; SLIT2: Slit homolog 2 (Drosophila); CINP1 FBN1: Fibrillin 1; SNCA: α-synuclein gene; SPG20: Spastic paraplegia-20; CYCD2: D-type cyclin gene; HIC1: Hypermethylated in cancer 1; RASSF1A: Ras association domain family 1 (isoform A); RB1: Retinoblastoma tumor suppressor; SRBC: Sheep red blood cells; THBD: Thrombomodulin; C9orf50: Chromosome 9 open reading frame 50; FRP2: Frizzled related protein 2; TPEF/HPP1: Transmembrane protein containing epidermal growth factor, follistatin domain/hyperplastic polyposis 1.

specificity for CRC detection during initial stages when

analyzing methylation status of circDNA^[67,69]. Although the evaluation some of these aberrant methylated genes may demonstrate better diagnostic results than the *SEPT9* analysis, their cost effectiveness, further technical improvement and low testing uptake issues impede their use within large-scale clinical trials^[70,93]. Thus, *SEPT9* as the most common blood-based methylation analysis biomarker holds promising example of sending on real life the laboratory methylation studies upon circDNA, for early CRC diagnosis of average-risk individuals.

circDNA pre-analysis considerations

As we stated before, analysis of *SEPT9* gene methylation could be subject of further technical advance^[93]. Therefore, it is reasonable to presume that several factors could play crucial role such as blood sampling and circDNA processing. It is well-established that blood-based circDNA could be extracted from both plasma and serum with the latter one exhibiting higher concentration of DNA^[94,95]. However there are studies suggesting that this high amount of DNA in serum reflects the *in vitro* lysis of leucocytes when the procedures of coagulation and/or fibrinolysis take place^[95]. Another theory highlights the significant effect that chemicals differences between serum and plasma have during DNA extraction^[96]. Other factors that researches should take into account are: The interval time of blood drawn and centrifugation; the sample storage modality; the anticoagulant used; temperature; and the plasma-based DNA isolation protocol^[96]. All these parameters exhibit enormous significance as concerns the efficiency and quality of circDNA analysis, illustrating the reliability that newer methods of circDNA analysis, should have.

CONCLUSION AND FUTURE ASPIRATIONS

Colorectal cancer is one of the deadliest malignancies to date even though various techniques are available to prevent and detect its emergence. Although these preventive modalities (sigmoidoscopy, colonoscopy, FOBT, FIT) exhibit high CRC detection sensitivity and specificity, the acceptance rate among population remains low. In parallel, the rapid progression of molecular biology has revealed new translational research fields related to discovery of potential CRC biomarkers in body "liquid fluids". These markers evaluate the fragments of DNA, RNA or proteins in the blood or feces demonstrating an increasingly cost-effective and sensitive way to detect premalignant modification of genome in individuals on average risk for CRC development. Thus, with this review we tried to highlight those circDNA blood-based biomarkers that offer an easy, cost-effective and with minimal invasiveness diagnosis of colonic neoplasia (Table 2). We believe that this research demonstrate in depth the need for further studies to be done which should be large randomized and will try to evaluate or elucidate the clinical value of all these new proposed screening tests which

could be combined the older ones as a critical strategy to improve quality of the existing life expectancy as well as to advance the latter one.

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Basic Study

Effect of *Clostridium perfringens* enterotoxin on gastric cancer cells SGC7901 which highly expressed claudin-4 protein

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Abstract

AIM

To investigate the effects of *Clostridium perfringens* enterotoxin (CPE) on gastric cancer cells which highly expressed claudin-4 (CL4) protein.

METHODS

In this study, we detected expression of CL4 protein in different gastric cancer cell lines. Then, we investigated the effects of CPE on SGC7901 cells which highly expressed CL4 protein and the effects of CPE on subcutaneous tumor in nude mice models.

RESULTS

CL4 are highly expressed in SGC7901 cells. CPE expressed

significant cytotoxicity in SGC7901 cells. Suppression of CL4 expression significantly decreased CPE-mediated cytotoxicity. CPE also inhibited tumor growth in subcutaneous tumor xenograft models.

CONCLUSION

CPE showed CL4 mediated cytotoxicity on gastric cancer cells SGC7901 and inhibited tumor growth in nude mice models.

Key words: Gastric cancer; *Clostridium perfringens* enterotoxin; Claudin-4 protein; Cytotoxicity; Tight junction

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Core tip: This study firstly investigated the effects of *Clostridium perfringens* enterotoxin (CPE) on gastric cancer cells SGC7901, and indicated CPE's potential effect in gastric cancer therapy.

Liang ZY, Kang X, Chen H, Wang M, Guan WX. Effect of *Clostridium perfringens* enterotoxin on gastric cancer cells SGC7901 which highly expressed claudin-4 protein. *World J Gastrointest Oncol* 2017; 9(4): 153-159 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i4/153.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i4.153>

INTRODUCTION

Gastric cancer is the second leading cause of cancer-related death through the world^[1]. System therapy including radical surgery, adjuvant chemotherapy, biology therapy and so on. However, 5 year survival rate in advanced gastric cancer patients was still low^[2,3].

Tight junction is the important structure between epithelial cells maintaining the cell polarity and membrane integrity^[4]. Tight junction are formed by some tight junction proteins including occludin, claudins, ZO-1 and so on^[5]. Recently, some studies show claudin-4 (CL4) protein plays a crucial role in tumor's proliferation, transformation, and metastasis^[6,7]. CL4 protein are highly expressed in many kinds of malignant tumors, such as ovarian cancer^[8]. In 2015, Liu *et al*^[9] reported that overexpression of CL4 protein was associated with progress of gastric cancer and poor prognosis of gastric cancer patients. More and more researches indicated that CL4 may be an emerging target for cancer therapy.

Clostridium perfringens enterotoxin (CPE), a 35-kDa single polypeptide comprised of 319 amino acids, could bind with CL4 and formed CPE-CL4 complex. CPE-CL4 complex induced resultant pore formation on cell membranes of epithelial cells, and caused cell apoptosis via the influx of Ca²⁺ into the cell^[10]. In pancreatic cancer cell lines HPAC cells, CPE showed a dose-dependent cytotoxic effect^[11]. In ovarian tumors, CPE also showed a dose-dependent cytotoxic effect *in vitro*. CPE significantly

inhibited tumor growth and progression in SCID mouse xenografts of human ovarian cancer^[12]. However, little was known about the effect of CPE on gastric cancer cells.

In this study, we assessed the expression of CL4 protein in different gastric cancer cell lines. Then we investigated the effects of CPE on SGC7901 cells which highly expressed CL4. In addition, we observed CPE effects on subcutaneous tumor growth of gastric cancer cell SGC7901 in nude mice model.

MATERIALS AND METHODS

Antibody

Goat polyclonal antibodies against CL4 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Rabbit polyclonal antibodies were obtained from Abcam (Cambridge, MA, United States). The secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-goat immunoglobulin (Ig) G (ZSGB-BIO, Beijing, China), Alexia Flour 488 (green)-labeled donkey anti-goat IgG (abcam, Cambridge, CA, United States).

Cell lines and cell culture

The human gastric cancer cell line SGC7901, MKN45, AGS, MGC803, BGC823 and HGC27 were used to assess the expression of CL4 protein. Colon cancer cell line Caco-2 was considered as CL4 positive control. Normal gastric epithelium cell line GES-1 was considered as negative control. All these cell lines were obtained from Cell Bank, Shanghai Institutes for Biological Sciences. All cells were maintained in RPMI-1640 with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin and cultured at 37 °C in a humidified 5% CO₂ atmosphere.

Western blot

Cells were cultured in 25-mm² Tissue Culture Flasks. Total protein was extracted by the Protein extraction kit (KeyGEN BioTECH, Shanghai, China). Twelve percent SDS-PAGE was used for electrophore. After completely separated, the target protein was transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore). The membrane was saturated with PBS containing 4% skim milk, and then incubated for one night at 4 °C with primary antibodies (diluted 1:1000) in PBS. After rinsing in PBS containing 0.1% Tween 20, the membrane was incubated for 2 h at room temperature with HRP-conjugated anti-rabbit or anti-goat IgG (diluted 1:10000) in PBS. It was then rinsed again, and finally reacted using an immobilon western chemiluminescent HRP substrate (Immobilon; Millipore). Signals in immunoblots were quantified using Quantity One-4.4.0 (Bio-Rad).

Preparation of CPE

The DNA sequence of CPE was synthesized and amplified by polymerase chain reaction (PCR) and subcloned into

vector pET28a, and the sequence was transfected to *Escherichia coli*. The CPE protein expression was induced by isopropyl- β -D-thiogalactoside (IPTG) and purified by Ni-IDA. This process was made by the company of Biogot technology (Nanjing, China).

Cytotoxicity assay

Cells were grown on 96-well plates to reach confluent density and incubated for 24 h with either the vehicle or CPE. Then 20 μ L 5 mg/mL methylthiazolyldiphenyl-tetrazolium bromide (MTT) was added to each well and incubated for 4 h. One hundred and fifty microlitre dimethyl sulfoxide was added to every well after the supernatant was wiped off. Then the plates were wobbled for 10 min. The optical density at the wavelength of 490 nm (OD₄₉₀) was detected with a microplate reader ELX800 (BioTek, VT). The inhibition ratio was calculated by formula $[\text{OD}_{490} (\text{CPE group}) / \text{OD}_{490} (\text{control group})] \times 100\%$. The final results were the average of 3 times and the image was made by OriginPro 9.2 (OriginLab).

RNAi and transfection

Stealth siRNA duplex oligonucleotides against human CL4 were synthesized by Invitrogen. The sequences were as follows: Sense (UCUGUUUUGUAAUUUAAGATT) and antisense (UCUUAAAUAACAAAACAGAAA). SGC7901 cells were transfected with siRNAs (final concentration was 10 nmol/L) or a Stealth RNAi negative control by using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's protocols.

Confocal microscope

Cells grown on coverslips were fixed in paraformaldehyde for 15 min at room temperature. After being covered with 10% BSA for 1 h, they were washed three times with PBS and incubated for a night at -4 °C with primary antibodies and rinsed again with PBS, followed by reaction for 2 h at room temperature with appropriate secondary antibodies. All samples were examined using a laser scanning confocal microscope (LSM710, Carl Zeiss, Jena, Germany). Photographs were recorded using a computer (Fujitsu) and ZEN 2009 (Carl Zeiss) and processed with Zeiss LSM Image Browser (Carl Zeiss) and Photoshop CS6 (Adobe).

Animal studies

SGC7901 cells (2×10^6 cells in 100 μ L of medium RPMI-1640) were subcutaneously injected into the inguinal region of 6-wk-old nude male mice (BALB/c, nu/nu, SLRC Laboratory Animal Technology Co, Shanghai, China). The mice were killed to get the tumor mass at 2 wk after injection. The tumor mass was cut into pieces with a diameter of 2 mm and planted into the subcutaneous of inguinal region of 6-wk-old nude male mice. After 2 wk the mice were divided into two groups (+CPE and -CPE), and 2 μ g of CPE in 100 μ L of saline, or 100 μ L of PBS was injected around the tumor every day for 10 d. The tumor volume (mm³) was calculated

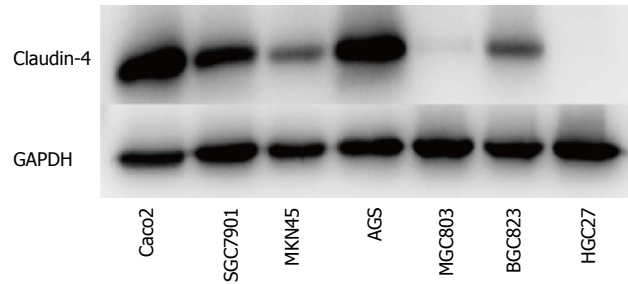


Figure 1 Expression of claudin-4 protein in different gastric cancer cells.

by the formula $0.5 \times \text{long diameter (mm)}^2 \times \text{short diameter (mm)}$. On day 10, the tumors were removed and the diameters of tumor were measured. All aspects of the study were approved by the Animal Use and Care Committee of Nanjing Drum Tower hospital (Nanjing, China).

Statistical analysis

All measured values are presented as the mean \pm SD. Statistical significance of differences was evaluated using One-Way ANOVA analysis and LSD test. Repeated measures analysis of variance was used for evaluating the animal studies.

RESULTS

CL4 protein expression in gastric cancer cells

We firstly evaluated the expression level of CL4 protein in different gastric cancer cells (SGC7901, MKN45, AGS, MGC803, BGC823 and HGC27 cells). Colon cancer cell line Caco-2 which highly expressed CL4 protein was considered as positive control cell. We found CL4 protein was highly expressed in two types of cell lines: SGC7901 and AGS cells (Figure 1). Because AGS cells can not be used in nude mice models, we select SGC7901 cells in further study to investigate CPE effects.

CPE cytotoxicity in SGC7901 cells

We confirmed the CL4 level of gastric cancer cells SGC7901 and normal gastric epithelium cells GES-1 by western blot. The relative level of CL4 on GES-1 cells was significantly lower than SGC7901 cells (Figure 2A). Cytotoxicity of up to 56% was observed 24 h in SGC7901 cells after CPE treatment and the cytotoxicity of CPE (2, 4, 6, 8, 10 mg/L) showed significant differences ($P < 0.05$) compared with CPE (0.2 mg/L). However, CPE had no significant cytotoxic effects on GES-1 cells under the same conditions (Figure 2B).

Suppression of CL4 expression decreased CPE cytotoxicity

We used an RNAi approach to knock down the CL4 protein in SGC7901 cells. Cells were transfected with the siRNA against human CL4 and incubated for 48 h. Western blot analysis showed that the siRNA significantly reduced CL4 protein expression in SGC7901 cells (Figure

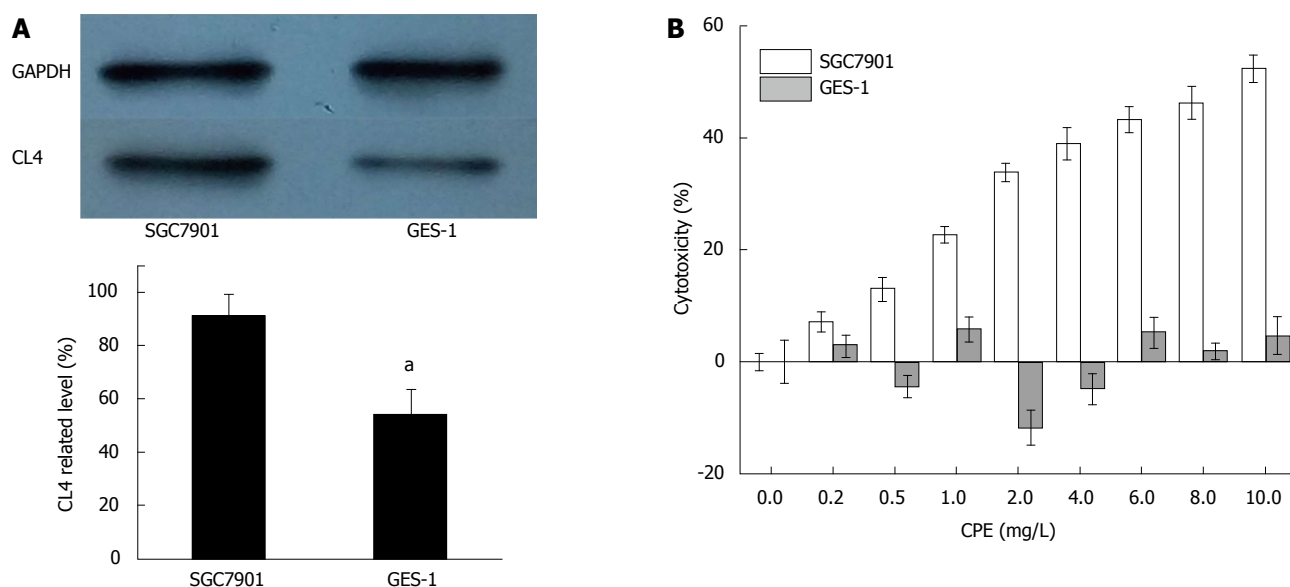


Figure 2 *Clostridium perfringens* enterotoxin effects on SGC7901 cells and GES-1 cells. A: CL4 expression in SGC7901 and GES-1 cells. The intensity levels represent as mean \pm SD ($n = 3$). $^aP < 0.05$ vs SGC7901 cells; B: Cells were treated with CPE (0, 0.2, 0.5, 1, 2, 4, 6, 8 and 10 mg/L) for 24 h, and the cytotoxicity of CPE was measured by MTT assay. Values represent as mean \pm SD ($n = 3$). CPE: *Clostridium perfringens* enterotoxin; CL4: Claudin-4.

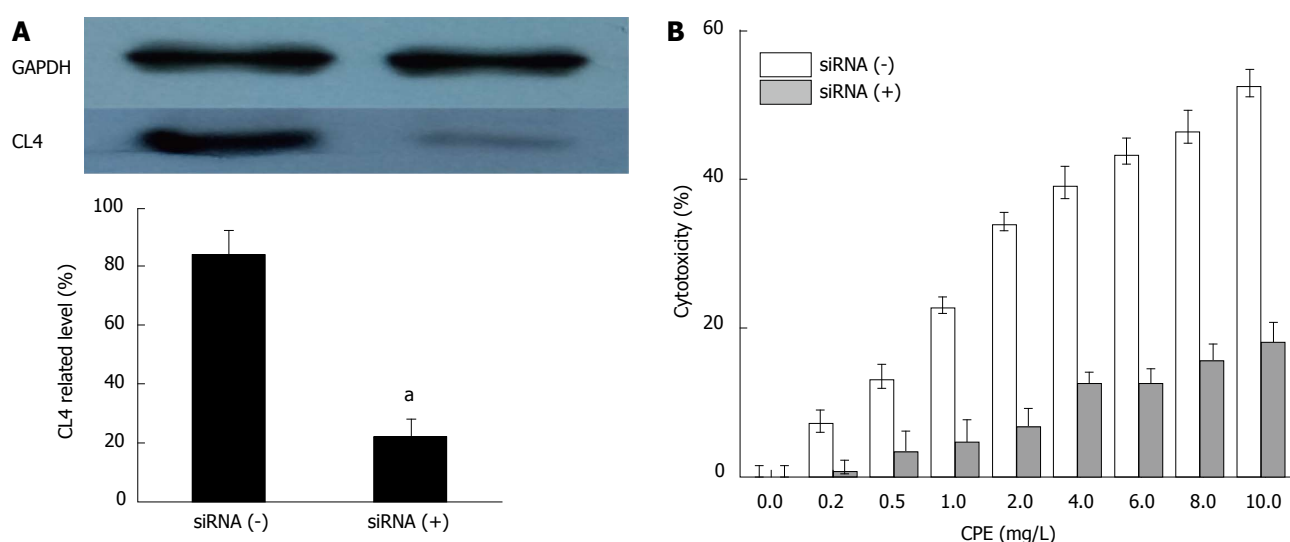


Figure 3 *Clostridium perfringens* enterotoxin effects on SGC7901 cells with siRNA transfection. A: SGC7901 cells were transfected with siRNA, and incubated for 24 h. CL4 expression in siRNA (-) and siRNA (+) cells were shown in A. $^aP < 0.05$ vs control group; B: The siRNA (-) and siRNA (+) SGC7901 cells were treated with different concentrations of CPE for 24 h. The MTT assay values represent as mean \pm SD ($n = 3$). CPE: *Clostridium perfringens* enterotoxin; CL4: Claudin-4.

3A). When the expression of CL4 was suppressed, CPE-mediated cytotoxicity was significantly decreased in SGC7901 cells according to MTT assay (Figure 3B).

CPE effect on CL4 expression in membrane

Cells were performed immunostaining and observed under a laser-scanning confocal microscope. The results showed that CL4 mainly expressed on the cell membrane in SGC7901 cells. After treatment with CPE (10 mg/L) for 24 h, CL4 protein expression in cell membrane were suppressed and partly translocated to cytoplasm (Figure 4A). However, CL4 expressed both on cell membrane and in cytoplasm in GES-1 cells. Cell membrane damage was not observed in GES-1 after CPE treatment (10 mg/L)

for 24 h (Figure 4B).

CPE inhibits tumor growth of SGC7901 xenografts in nude mice

To evaluate the cytotoxic effect of CPE *in vivo*, SGC7901 cells were used to establish the xenograft models. When the subcutaneous tumor diameter reached about 5-7 mm, the nude mice were randomly divided into two groups [CPE (+) group, $n = 7$; CPE (-) group, $n = 7$]. CPE (+) group was injected with CPE (2 mg in 100 μ L of PBS) around the tumors once a day for 10 d, while CPE (-) group was injected with PBS (100 μ L) around the tumors once a day for 10 d. Tumor volumes were measured on days 0, 5, and 10 after treatment. Mice were killed and

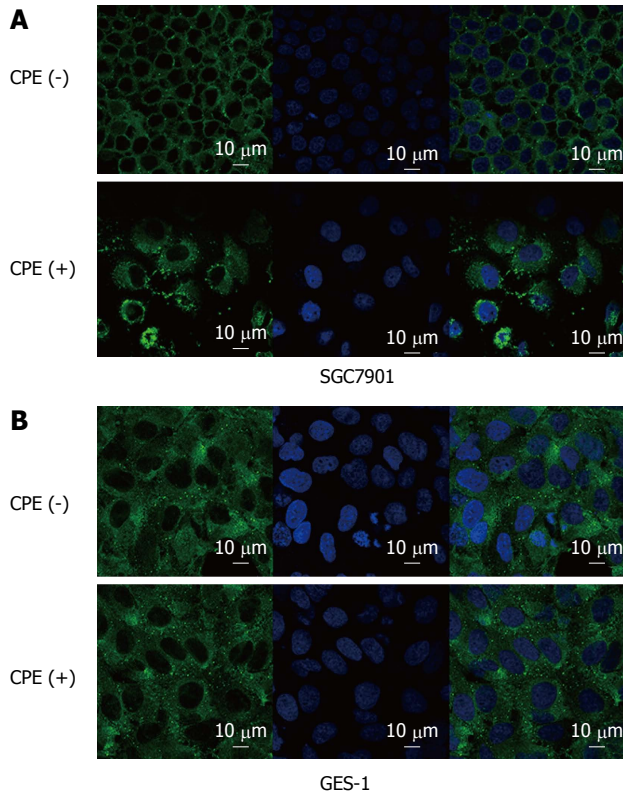


Figure 4 *Clostridium perfringens* enterotoxin effect on claudin-4 protein in cell membrane. SGC7901 Cells (A) and GES-1 cells (B) were subjected to CL4 immunostaining under a laser-scanning confocal microscope. Green strains illuminated CL4 protein expression. CPE: *Clostridium perfringens* enterotoxin; CL4: Claudin-4.

the tumors were removed after 10 d. The tumor tissues were shown in Figure 5A, CPE significantly suppressed tumor growth, and obvious reduction of tumor volume was observed in CPE (+) group compared with CPE (-) group (Figure 5B). However, in CPE (+) group, injection site skin necrosis and enteritis were also observed in 3 mice.

DISCUSSION

The receptors of CPE were mainly considered as CL3 and CL4 protein. CL4 has been found highly expressed in some gastric cancer tissues^[13-16]. Hung Jung found the expression rate of CL4 was 44.4% in gastric cancer tissues, and expressions of CL4 was significantly lower in cases with positive lymphatic invasion^[13]. Liang *et al.*^[14] found the expression of CL4 in normal stomach samples was only 15.9%. Maeda's study found that inhibiting the expression of CL4 significantly reduced the CPE toxicity, but inhibiting the expression of CL4 slightly increased the toxicity of CPE in prostate cancer^[17]. In this study, we also observed inhibiting CL4 expression significantly reduced CPE-mediated toxicity in gastric cancer cells. These results revealed CL4 protein could be a potential target agent in gastric cancer therapy.

Our study found that CPE almost had no significant toxicity on normal gastric epithelium cells GES-1 and

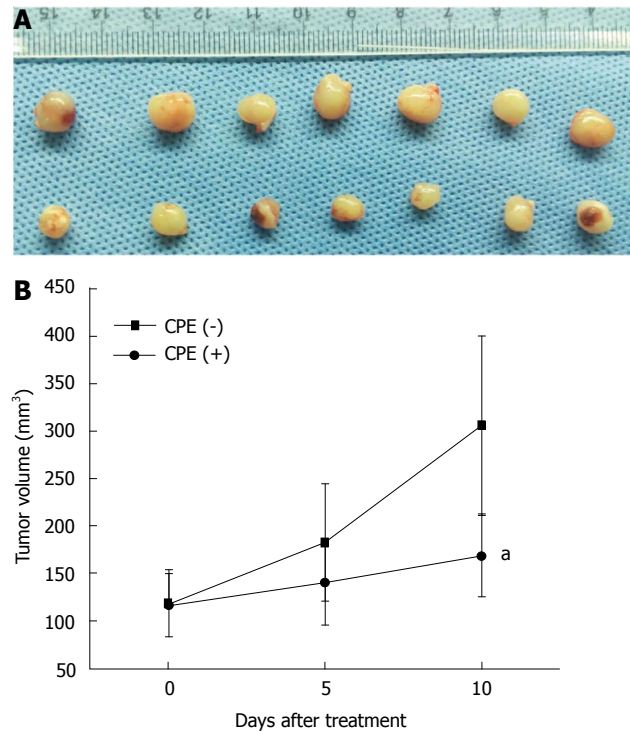


Figure 5 *Clostridium perfringens* enterotoxin effects on SGC7901 xenograft tumor in nude mice model. A: SGC7901 xenografts were randomly divided into two groups. Mice were killed on day 10 after CPE treatment and the tumors were measured; B: Obvious reduction of tumor volume was observed in CPE (+) group. The data present as mean \pm SD. ^a $P < 0.05$ vs CPE (-) group. CPE: *Clostridium perfringens* enterotoxin.

the laser confocal microscopy confirmed that CPE had little effects on membrane morphology in GES-1 cells. According to the former study, the toxicity of CPE was associated not only with CL4 expression, but also the subcellular localization of CL4. As the target of CPE, CL4 mainly distributed in the cell membrane in SGC7901 cells, but distributed both in cell membrane and cytoplasm in GES-1 cells. While, the overall expression of CL4 in GES-1 was significantly lower than SGC7901 cells according to the Western blot test. We speculated that little CL4 protein distributed in membranes in GES-1 cells. In addition, recent study found the formation of intact tight junction could alleviate the cytotoxicity of CPE^[17]. Studies also found solid tight junctions could be formed between GES-1 cells^[18]. These findings maybe explained the different effects of CPE on GES-1 cells and SGC7901 cells.

Tight junction plays a very important role in the proliferation, differentiation and cell polarity of epithelial cells^[19]. In our experiment, SGC7901 cell membrane was integrity and the size was substantially uniform. After CPE treatment, part of the cell membrane was not complete. Some nucleus split into smaller pieces after CPE treatment (10 mg/L). This phenomenon agreed with Smedley's study, which found CPE caused apoptosis at low concentrations while oncosis at high concentrations^[10].

Although CPE showed potential therapeutic effects

on some malignant tumors, there were still no clinical data or trials available. CPE's side effect limited its clinical application in tumor therapy. In this study, CPE injection site skin necrosis and enteritis were observed in 3/7 mice. Garcia *et al.*^[20] also found the rabbit's small intestine and colon were damaged after as little as a 1-h treatment with 50 µg/mL of CPE. These studies indicated that serious adverse effects should be considered in CPE-based cancer therapy. To overcome these disadvantages, some researchers cut off the N-terminal region of CPE which mainly cause cell death and then obtain C-terminal CPE (C-CPE) which mainly target to the cells. C-CPE is a smaller molecule without cytotoxicity but also combined with CL4 protein. C-CPE can disrupt the tight junction and increase paracellular permeability, enhance chemotherapy drugs to get into the cells^[21]. Li *et al.*^[22] observed the safety of the C-terminal of CPE and confirmed that injection of CL-targeted toxin injured the liver but not the kidney. To alleviate the side effect of C-CPE is highlight in future research.

In summary, this study investigated the effects of CPE on gastric cancer cells SGC7901. CPE showed CL4 mediated cytotoxicity on gastric cancer cells, and inhibited tumor growth in nude mice models. These results provide CPE may be a novel potential tool for gastric cancer's therapy. More studies need to be performed to overcome the limitation of CPE before its clinical application.

COMMENTS

Background

Clostridium perfringens enterotoxin (CPE) showed therapeutic effects on malignant tumors which highly expressed claudin-4 (CL4) protein. However, little was known about the effects of CPE on gastric cancer cells.

Innovations and breakthrough

In this study, the authors firstly investigated the effects of CPE on SGC7901 cells which highly expressed CL4 protein. CPE showed CL4 mediated cytotoxicity on gastric cancer cells SGC7901 and inhibited tumor growth in nude mice models.

Applications

These results provide CPE may be a novel potential tool for gastric cancer's therapy.

Peer-review

This is an interesting article reporting the therapeutic effect of CPE on gastric cancer cells (SGC7901 cells) and on a subcutaneous tumor in nude mice model.

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Observational Study

Bayesian adjustment of gastric cancer mortality rate in the presence of misclassification

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Abstract

AIM

To correct for misclassification error in registering causes of death in Iran death registry using Bayesian method.

METHODS

National death statistic from 2006 to 2010 for gastric cancer which reported annually by the Ministry of Health and Medical Education included in this study. To correct the rate of gastric cancer mortality with reassigning the deaths due to gastric cancer that registered as cancer without detail, a Bayesian method was implemented with Poisson count regression and beta prior for misclassified parameter, assuming 20% misclassification in registering causes of death in Iran.

RESULTS

Registered mortality due to gastric cancer from 2006 to 2010 was considered in this study. According to the Bayesian re-estimate, about 3%-7% of deaths due to gastric cancer have registered as cancer without mentioning details. It makes an undercount of gastric cancer mortality in Iranian population. The number and age standardized rate of gastric cancer death is estimated to be 5805 (10.17 per 100000 populations), 5862 (10.51 per 100000 populations), 5731 (10.23 per 100000 populations), 5946 (10.44 per 100000 populations), and 6002 (10.35 per 100000 populations), respectively for years 2006 to 2010.

CONCLUSION

There is an undercount in gastric cancer mortality in Iranian registered data that researchers and authorities should notice that in sequential estimations and policy making.

Key words: Misclassification; Bayesian method; Cause of death; Gastric cancer; Iran

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Core tip: In some mortality cases, causes of deaths are registered as causes that cannot or should not be considered as the underlying causes of death like cancer without mentioning the type. These cases are not included in the estimations of cause specific mortality rates and leads to under-estimate health risks and burden of disease. The aim of this study is to correct the misclassification of gastric cancer deaths in cancer without label group using a Bayesian method.

Hajizadeh N, Pourhoseingholi MA, Baghestani AR, Abadi A, Zali MR. Bayesian adjustment of gastric cancer mortality rate in the presence of misclassification. *World J Gastrointest Oncol* 2017; 9(4): 160-165 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i4/160.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i4.160>

INTRODUCTION

Cancer is one of the major health problems in the world and is the third cause of death (after cardiovascular disease and injuries) in Iran^[1]. Gastric cancer is a disease in which the cells of the inner lining of the stomach start to divide abnormally and uncontrollably, that forming a mass called tumor^[2]. Gastric cancer is the seventh cause of all deaths in Iran and is the first cause of cancer death in Iranian men and the second cause of cancer death (after breast cancer) in Iranian women^[3]. The mortality of gastric cancer is high because this cancer does not show symptoms in early stages and diagnosed when the cancer is in its final stages^[4].

Burden of disease is used to evaluate the health

status of a country and determining priority of risk factors in order to setup cancer control programs. Cancer registry data are important to estimate the burden of disease, monitoring the screening programs effects, early diagnostics and other prognostic factors, and can be used to guide policy makers to appropriate cancer prevention programs. Among medical indices, mortality is a familiar projection to assess the burden of diseases. But achieving this aim requires a reliable death registry systems that reports death statistics accurately and completely^[5-7]. In Iran, among four vital events (births, marriages, divorces and mortality) which were registered by the National Organization for Civil Registration (NOCR), mortality was the worst in quality. There was some progress in registering deaths but some problems like delayed registration and inaccurate recording of causes of death remained until 2002, that Ministry of health and medical education Deputy of Research and Technology, started up a system to record the causes of deaths. This system did not allow to delayed deaths registry, but the causes of death were susceptible to information bias due to misclassification^[8]. Most high-income and many middle-income countries have a complete vital registration system in which the majority of deaths get a death certificate completed by a physician^[9]. But still, a number of causes of death in the process of completing death certificates and the coding of underlying cause of death based on standardized international rules, remains challenging^[10-13]. In some cases, especially in developing countries, the cause of death is recorded with error^[14,15]. For example if a death due to gastric cancer being labeled as a death due to any other cause, the misclassification error in outcome is occurs. Misclassification error makes the registered data inaccurate and often leads to major problems like biased estimates of burden and health risks in epidemiological analysis^[16,17].

According to the Iranian death registry, about 15% to 20% of death statistics are recorded in misclassified categories such as cardiopulmonary arrest, old age without dementia, septicemia, unknown, cancer without mention of details, and other ill-defined conditions. Murray and Lopez in 1996, for the first time, introduced the term "garbage coding" for assigning deaths to causes that are not useful for public health analysis of cause-of-death data^[18-21].

In developing countries like Iran that registration is not completely accurate, statistical methods can be very helpful to overcome this problem. Two statistical approaches are recommended to deal with misclassification; first is using a small valid sample and extending the results to the population^[22] and the second is Bayesian analysis which is a flexible method that makes the possibility of combining the prior information regarding the subset of the parameters with the observed data to achieve a posterior distribution which will be the basis of inferences to correct the statistics. Bayesian models also can easily accommodate unobserved variables such as an individual's true information in the presence of Misclassification error^[23]. The aim of this study is to use

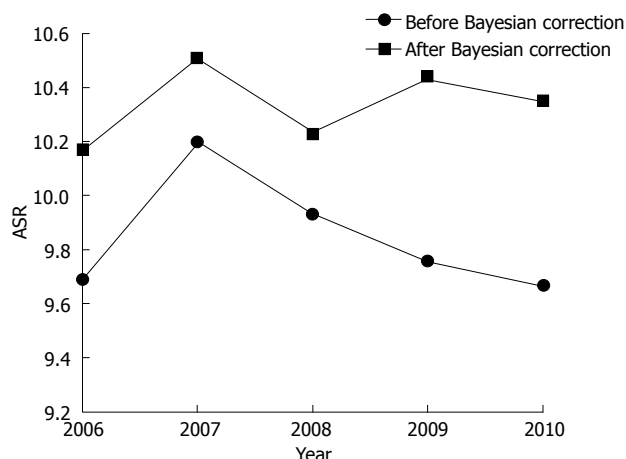


Figure 1 Age standardized rate of gastric cancer mortality in Iran from 2006 to 2010, before and after Bayesian correction of misclassification in causes of death. ASR: Age standardized rate.

Bayesian method to estimate the rate of misclassification that occurs by registering cancer (with no label) as the cause of death instead of deaths that have occurred because of gastric cancer in Iran's cancer registry system.

MATERIALS AND METHODS

Mortality rates due to gastric cancer and also cancer without label from 2006 to 2010 are extracted from Iranian annual of death statistics which reported annually by Iran's Ministry of Health and Medical Education, in two sex groups (male and female) and four age groups (under 15 years - 15 to 49 years - 50 to 69 years - 70 years and more).

To reassign deaths from garbage codes to valid causes, the approach can be divided into three steps: The first is identifying garbage codes. The second is identifying the target causes where the deaths assigned to a garbage code should in principle be reassigned to; for example if a death cause is registered as cancer and the type of cancer is not mentioned, we face with a garbage code that should be reassigned to a specific cancer. The third step is choosing the fraction of deaths that are assigned to the garbage code that should be reallocated to the target cause^[13]. In this study we consider cancer without label as garbage code because cancer with no label is most likely to be registered as cause of death instead of a specific cancer like gastric cancer. The data were entered to the Bayesian model by two vectors $y_1 = [y_{11}, y_{21}, \dots, y_{r1}]$ for gastric cancer and $y_2 = [y_{12}, y_{22}, \dots, y_{r2}]$ for cancer without label. Both y_1 and y_2 are count data and follow the Poisson distribution. The subscript r shows the number of covariate patterns that is made by age and sex group combinations. θ is considered to be the probability of incorrectly register a mortality from gastric cancer as mortality due to cancer without label group. To perform Bayesian inference, an informative beta prior distribution was assumed for the misclassified parameter, *i.e.*, $\theta \sim \text{beta}(a, b)$. The initial value for the parameter of beta distribution

are taken to be $a = 20$ and $b = 80$, based on Iranian annual cancer registration reports. Since θ (misclassified parameter) is an unknown parameter, a latent variable approach was employed to simplify the full conditional models; considering $U_i | \theta, y_1, y_2 \sim \text{Binomial}(y_{i2}, P_i)$ as the number of counts from the first group that are incorrectly labeled as being in the misclassified group that $P_i = (\lambda_{i1}\theta) / (\lambda_{i1}\theta + \lambda_{i2})$, finally the posterior distribution appears in the following form; $\theta | U_i, y_1, y_2 \sim \text{Beta}(\sum U_i + a, \sum y_i + b)$. The misclassified parameter is estimated using a Gibbs sampling algorithm and averaging of the outcome. Analyses were done using R software version 3.2.0.

RESULTS

Mortality data consisting of all deaths due to gastric cancer from 2006 to 2010 were considered in this study. Age standardized rate (ASR) of gastric cancer mortality was 9.69 per 100000 populations in 2006, 10.2 per 100000 populations in 2007, 9.93 per 100000 populations in 2008, 9.76 per 100000 populations in 2009 and 9.67 per 100000 populations in 2010 respectively. According to the Bayesian estimation, in year 2006, there was between 3% to 7% misclassification in registering cause of death as cancer without mentioning details while the underlying cause of death has been gastric cancer. The estimated percent of misclassification based on implemented Bayesian method for year 2006 to 2010 is shown in Table 1. This percent were subtracted from deaths that had registered as cancer without mentioning details and added to the number of deaths due to gastric cancer. The age standardized rate per 100000 populations for gastric cancer was estimated to be 10.17 in 2006, 10.51 in 2007, 10.23 in 2008 10.44 in 2009 and 10.35 in 2010, after Bayesian correction respectively. The age standardizes rate of gastric cancer before and after Bayesian correction for 2006 to 2010 is visualized in Figure 1. The number of gastric cancer death before and after Bayesian correction of misclassification for years 2006 to 2010 is shown in Table 1 and its trend is shown in Figure 2.

DISCUSSION

Iran's death registry is subject to misclassification in reporting the underlying cause of death. About 3%-7% of deaths due to gastric cancer are registered as cancer without mentioning the type of cancer. After correcting misclassification error in death registry data, the number of deaths due to gastric cancer and its age standardized rate were increased. Gastric cancer crude mortality count in Iran had an increasing trend from year 2006 to 2010 except for 2008 that might be because of incompleteness of data; but the age standardized rate of gastric cancer was decreasing from year 2007 onward (except for 2008). About two-thirds of gastric cancer occurs in developing countries^[24-27] and its rates are generally about twice as high in men as in women^[28]. The age standardized rate (ASR) of gastric cancer incidence and

Table 1 Misclassified parameter and the number of gastric cancer death before and after Bayesian correction and percent of increase in number of deaths after Bayesian correction in Iran's death registry 2006-2010

Year	Misclassified parameter	Before Bayesian correction			After Bayesian correction		
		Male	Female	Total	Male	Female	Total
2006	5%	3887	1642	5529	4081	1724	5805
2007	3%	4001	1690	5691	4121	1740	5861
2008	3%	3912	1652	5564	4029	1702	5731
2009	7%	3907	1650	5557	4180	1766	5946
2010	7%	3944	1665	5609	4220	1782	6002

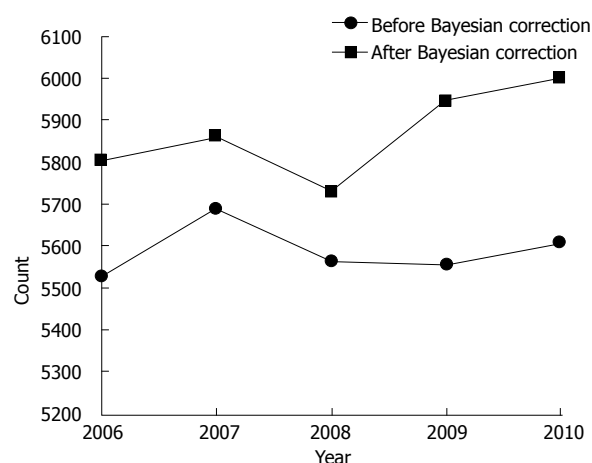
Table 2 Incidence and mortality age standardized rates per 100000 populations due to gastric cancer for some continents, reported by GLOBOCAN 2012

Continent	Incidence ASR	Mortality ASR
World	12.1	8.9
Asia	15.8	11.7
Europe	9.4	6.9
South America	10.3	8.5
North America	4.0	2.1
Africa	3.8	3.5

ASR: Age standardized rates.

mortality per 1000000 populations based on GLOBOCAN report 2012 is shown in Table 2. The rates show that the ASR of gastric cancer incidence (15.8 per 100000) and also the ASR of gastric cancer mortality (11.7 per 100000) is highest in Asia compared to other continents; It is moderate in Europe and South America and lowest in Northern America and most parts of Africa^[3,28].

The age standardized rates of incidence and mortality per 100000 populations in different regions of Asia based on GLOBOCAN report 2012 are shown in Table 3. The incidence and mortality rates are also higher in Eastern Asia in comparison with other Asian regions. This region includes China, Japan and South Korea, that are three countries with the highest gastric cancer incidence and mortality rates^[29]. Gastric cancer is the most frequently diagnosed form of cancer in Iran^[30], with incidence rate 15.3 per 100000 and mortality rate 12.9 per 100000 populations based on GLOBOCAN report 2012^[3]. A steady decline has been observed in gastric cancer incidence and mortality rates in the most of countries in Northern America and Europe since the middle of the 20th century^[31,32]. In recent years similar decreasing trends have been noted in areas with high rates of gastric cancer history, including some countries in Asia (Japan, China, and South Korea), Latin America (Colombia and Ecuador), and Europe (Ukraine)^[33]. This reduction maybe due to improved sanitation and antibiotics and consequently reduction in chronic *H. pylori* infection^[34]. Although the age-adjusted rates have been decreased, it is estimated to have a substantial rise in the crude rates between the years 2000 to 2020 because of the increasing the size and age of the world population, especially in developing countries^[35,36].

**Figure 2** Crude number of gastric cancer mortality in Iran from 2006 to 2010, before and after Bayesian correction of misclassification in causes of death.

Gastric cancer is a major health problem in the world, especially in Asia. So it is needed to make appropriate policy making for allocation of resources for gastric cancer control and prevention. To achieve this aim an accurate registry system is needed, while there are some misclassifications in registering causes of death especially in developing countries^[14,15]. Misclassification of causes of death has been a concern in cancer trends analysis and researches on cancer epidemiology for decades^[14]. Misclassification error leads to under-estimation of cause specific mortality rates and consequently under-estimation in burden of disease and influences the policy makings and health risk prioritizations^[10-12,37]. In the study of Khosravi *et al*^[38], validated data from hospital death was used to measure the impact of misclassification on rates of cardiovascular disease mortality. But they didn't employ Bayesian method. Bayesian approach has received much attention to correct for misclassification in mortality data. Whittemore and Gong^[39] used a Bayesian approach to estimate cervical cancer mortality rates and Sposto *et al*^[40] developed maximum likelihood method for assessing the effect of diagnostic misclassification on non-cancer and cancer mortality in atomic-bomb survivors. Stamey *et al*^[41] provided a Bayesian approach, which extends the models introduced by Whittemore and Gong^[39] and Sposto *et al*^[40]. They assume that the misclassification parameters are unknown. They used the prior information on the misclassification parameters instead of using valid

Table 3 Incidence and mortality age standardized rates per 100000 populations due to gastric cancer for different regions of Asia, reported by GLOBOCAN 2012

Region	Incidence ASR	Mortality ASR
Eastern Asia	24.2	16.5
Western Asia	9.5	8.1
South-Central Asia	6.7	6.1
South-Eastern Asia	6.0	5.3

ASR: Age standardized rates.

data. They applied their Bayesian approach for estimating the number of deaths due to cancer and non-cancer after correcting for misclassification in registering causes of deaths among survivors of Hiroshima and Nagasaki after atomic bombings^[41]. Pourhoseingholi *et al*^[42] extended the models proposed by Stamey *et al*^[41] to re-estimate the rates of cause specific deaths in cancer registry data after correcting for misclassification^[25,42,43]. Based on his study on gastric cancer mortality in Iranian population from 1995 to 2004, there were between 30%-40% misclassification in recording deaths due to gastric cancer^[44]. The current study reveals that the accuracy of death registration in Iran is getting better in recent years.

In conclusion there is an undercount of gastric cancer mortality in Iranian registration system. Because of misclassification error in registering causes of death. Although it seems that the misclassification rate has been reduced, it still exists as a major problem. So, policy makers who use mortality data to determine priorities for disease control and prevention, should notice to this underreported data and registration of causes of deaths should be done more accurately. Increase in data accuracy, requires more expert staffing, refining foundations, and powerful hardware and software resources^[45]. In the absence of valid data, Bayesian approach is a good and flexible alternative to reduce the effects of Misclassification in registered cancer mortality data.

COMMENTS

Background

Mortality data registries are subject to misclassification; because some deaths assigned to causes that cannot be considered as underlying death cause. For example if mortality due to a special cancer be registered as cancer without mentioning the type of cancer, misclassification error occurs. The aim of this study is to estimate the rate of misclassification in registering deaths due to gastric cancer in cancer without label group using a Bayesian method and re-estimate the rate of gastric cancer mortality in Iran.

Research frontiers

In Iran, death registries data is subject to misclassification. Reviewing the medical records or verbal autopsy as a practical solution for misclassification is time consuming. The hotspot of this study is using the Bayesian method for estimating the rate of misclassification in registering causes of death, which is rapid and cost-effective.

Innovations and breakthroughs

By using the Bayesian method, it is not needed to valid the data for estimating the rate of misclassification. Data validation is very costly and time consuming

and in many cases it is not possible to obtain valid data. For implementing the Bayesian method only prior information about the misclassification rate is enough.

Applications

Since registered mortality data is used for health policy making and estimating the burden of disease, after correcting the misclassification in death registry system, more precise estimates of death rates and cause specific burden of disease will be achieved. Consequently there will be a better planning for disease control and prevention.

Terminology

Misclassification is lack of agreement between the observed value and the true value in categorical data. Bayesian method is one of the statistical approaches that assign a distribution or a probability to events or parameters based on previous experience or an expert's idea and revise those probabilities and distributions after obtaining experimental data with applying Bayes' theorem.

Peer-review

This is an interesting research.

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Observational Study

Macroscopic appearance of Type IV and giant Type III is a high risk for a poor prognosis in pathological stage II / III advanced gastric cancer with postoperative adjuvant chemotherapy

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Abstract

AIM

To evaluate whether a high risk macroscopic appearance (Type IV and giant Type III) is associated with a dismal prognosis after curative surgery, because its prognostic relevance remains elusive in pathological stage II / III (pStage II / III) gastric cancer.

METHODS

One hundred and seventy-two advanced gastric cancer (defined as pT2 or beyond) patients with pStage II / III who underwent curative surgery plus adjuvant S1 chemotherapy were evaluated, and the prognostic relevance of a high-risk macroscopic appearance was examined.

RESULTS

Advanced gastric cancers with a high-risk macroscopic appearance were retrospectively identified by preoperative recorded images. A high-risk macroscopic appearance showed a significantly worse relapse free survival (RFS) (35.7%) and overall survival (OS) (34%) than an average risk appearance ($P = 0.0003$ and $P < 0.0001$, respectively). A high-risk macroscopic appearance was significantly associated with the 13th Japanese Gastric Cancer Association (JGCA) pT ($P = 0.01$), but not with the 13th JGCA pN. On univariate analysis for RFS and OS, prognostic factors included 13th JGCA pStage ($P < 0.0001$)

and other clinicopathological factors including macroscopic appearance. A multivariate Cox proportional hazards model for univariate prognostic factors identified high-risk macroscopic appearance ($P = 0.036$, HR = 2.29 for RFS and $P = 0.021$, HR = 2.74 for OS) as an independent prognostic indicator.

CONCLUSION

A high-risk macroscopic appearance was associated with a poor prognosis, and it could be a prognostic factor independent of 13th JGCA stage in pStage II/III advanced gastric cancer.

Key words: Macroscopic feature; Gastric cancer; Type IV; Giant type III; Stage II/III

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Core tip: In this study, we for the first time clarify the clinicopathological relevance of the macroscopic high risk patients with pathological stage II/III gastric cancer who underwent curative surgery with postoperative S1 adjuvant chemotherapy in Japan.

Yamashita K, Ema A, Hosoda K, Mieno H, Moriya H, Katada N, Watanabe M. Macroscopic appearance of Type IV and giant Type III is a high risk for a poor prognosis in pathological stage II/III advanced gastric cancer with postoperative adjuvant chemotherapy. *World J Gastrointest Oncol* 2017; 9(4): 166-175 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i4/166.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i4.166>

INTRODUCTION

Gastric cancer is the fifth most common malignancy and the third leading cause of cancer-related deaths worldwide^[1]. Advanced gastric cancer with depth of invasion of T2 or beyond continues to show unsatisfactory survival outcomes despite progress in multidisciplinary therapy, especially for postoperative S1 adjuvant therapy^[2,3], while early gastric cancer is largely a curable disease^[4,5]. Among advanced gastric cancers, macroscopic features and patient age were recently proven to be simple but the most potent independent prognostic factors^[6]. Type IV and large type III gastric cancer have the most dismal prognosis^[6-8].

The gastric cancer section of the Japan Clinical Oncology Group (JCOG) has also classified advanced gastric cancer into macroscopic high risk and average risk to conduct clinical trials to propose novel multimodal treatment strategies. Giant type III (designated as 8 cm in length or greater) and type IV gastric cancer are being proposed as high-risk gastric cancer with dismal prognoses, for which neoadjuvant chemotherapy of cisplatin/S1 (CS) may be promising as a novel therapeutic strategy^[9]. This strategy may be successful because of the clinical success of neoadjuvant chemo-

therapy for gastric cancer in the Western world, where neoadjuvant chemotherapy with epirubicin/cisplatin/5-fluorouracil (ECF) improved progression free survival (PFS) and overall survival (OS) better than surgery alone in aggressive advanced gastric cancer; gastric cancer in Western countries has shown a more aggressive phenotype than in Eastern countries^[10].

Macroscopic features have been repeatedly reported to be a prognostic factor independent of stage as earlier described^[6,7], but there have been no investigations of their relevance in advanced gastric cancer patients with pathological stage II/III who underwent curative gastrectomy together with postoperative S1 adjuvant chemotherapy. In this study, the clinicopathological relevance of macroscopic high-risk with pathological stage II/III gastric cancer in patients who underwent curative surgery with postoperative S1 adjuvant chemotherapy was examined for the first time.

MATERIALS AND METHODS

Registration of patients

Between January 1, 2000, and December 31, 2010, 1673 patients underwent gastrectomy for gastric adenocarcinoma in the gastrointestinal surgery division, Kitasato University Hospital. A total of 396 patients with 13th Japanese Gastric Cancer Association (JGCA) stage II/III advanced gastric cancer underwent curative gastrectomy with D1-D2 lymph node dissection, and 67 underwent neoadjuvant chemotherapy or postoperative chemotherapy other than S-1 as previously reported^[11-13]. Advanced gastric cancer was defined as pathological T2 (13th JGCA stage) or beyond, and pT1 gastric cancers were excluded from this study even when they were pathological stage II. Older age, defined as 67 years of age or older was used from a prognostic point of view from the previous reports^[11]. Among the 329 patients with pStage II/III, 172 agreed to undergo adjuvant S-1 therapy after curative resection. The 172 patients who underwent adjuvant S-1 chemotherapy after surgery for at least one day were registered in the S-1 group. The clinicopathological features of the 172 patients in this study were investigated.

We participated in the ACTS-GC trial^[2], and started postoperative adjuvant use of S-1 for pStage II/III gastric cancer from October, 2001. Since 2007, when the interim analysis of the trial results was disclosed and recommended annual S-1 therapy after curative operation^[2], we recommended S-1 postoperative adjuvant therapy to patients with 13th JGCA pStage II/III advanced gastric cancer.

Among the 172 patients, D1 lymph node dissection ($n = 26$) was performed for various reasons: preoperative diagnosis of clinical T1 cancer ($n = 12$), omitted D2 dissection in the operative views (surgical T1) during the surgery ($n = 4$), omitted D2 dissection because of systemic complications ($n = 6$), surgery of remnant stomach cancer ($n = 3$); and elderly ($n = 1$).

The dose of S-1 was determined based on body

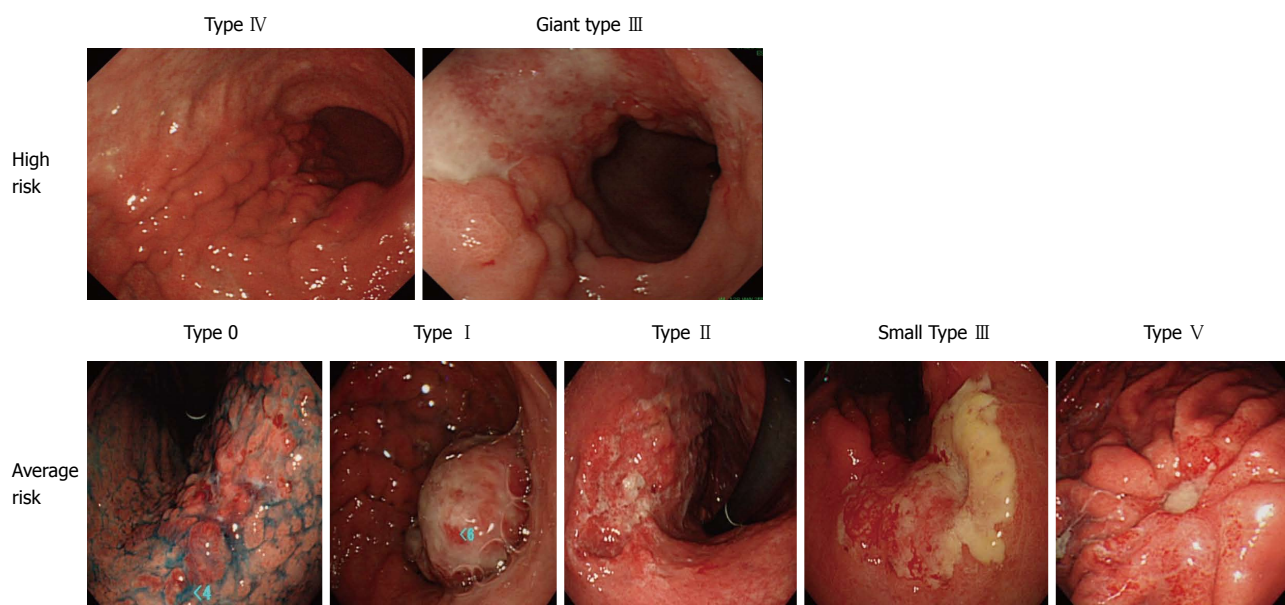


Figure 1 Representative gastroendoscopy images of advanced gastric cancer by macroscopic classification. Upper panels include high-risk macroscopic features of type IV (left) and giant type III (right). Lower panels include average risk macroscopic features of type 0, type I, type II, small type III, and type V (in order from left to right).

surface area: $< 1.25 \text{ m}^2$ (80 mg daily); $\geq 1.25 \text{ m}^2$ but $< 1.50 \text{ m}^2$ (100 mg daily); $\geq 1.50 \text{ m}^2$ (120 mg daily). The adjuvant S-1 chemotherapy regimen was administered for 4 wk followed by 2 wk of rest. This 6-wk cycle was repeated during the first year after surgery. Toxicity of chemotherapy was assessed using Common Toxicity Criteria of the National Cancer Institute, version 4.0 (NCI-CTC)^[14]. If patients had hematologic toxic effects of grade 3 or 4 or nonhematologic toxic effects of grade 2, 3 or 4, their daily dosage was reduced, or their treatment was postponed or stopped according to each physician's judgment.

13th JGCA stage

In the present study, the 13th JGCA stage classifications were used^[15], because ACTS-GC was established based on this staging system. In the 13th edition, the T category is classified into four categories: T1, the depth of invasion is mucosal or submucosal; T2, the depth of invasion is muscularis propria or subserosa; T3, the depth of invasion is serosa exposed; and T4, the depth of invasion is infiltrating into other organs. On the other hand, the status of lymph node metastasis is classified into four categories according to the anatomical classification of the involved lymph nodes. The descriptions are as follows: N0, no evidence of lymph node metastasis; N1, metastasis within the first tier of lymph nodes; N2, metastasis within the second tier of lymph nodes (extra-perigastric regional lymph nodes); and N3, metastasis to the third tier of lymph nodes (extra-regional lymph nodes). The latest (7th) UICC TNM stage is shown for reference purposes.

Clinicopathological factors

Macroscopic features were retrospectively determined by

gastro-endoscopy based on the 13th JGCA classification^[15] in combination with computed tomography (CT). Type 0, mucosal or submucosal; Type I, polypoid; Type II, fungating, ulcerated with sharp raised margins; Type III, ulcerated with poorly defined infiltrative margins; Type IV, infiltrative, predominantly intramural lesion, poorly demarcated; Type V, unclassified features. Representative tumors were shown in Figure 1. Giant type III was defined by its maximal diameter (8 cm or greater) assessed by upper gastrointestinal (UGI) barium contrast series as recently described^[8].

All histologic and clinicopathological factors were assessed independently and blindly by any of 20 well trained histopathologists. Lymphatic invasion (ly) and vascular invasion (v) were defined as ly0, 1, 2, and 3 and v0, 1, 2, and 3 by infiltrative grade. Histologically, there are two major types of gastric adenocarcinoma (Lauren's classification). In this study, cancers were classified into diffuse type (por1, por2, sig, muc) and intestinal type (pap, tub1, tub2).

Statistical analysis

Cumulative 5-year OS was estimated by the Kaplan-Meier method, and statistical differences were tested by the log rank test. OS was measured from the date of surgery to the date of death or the last follow-up. Fatal cases in the analysis of OS included those who died from causes other than gastric cancer. Cumulative 5-year relapse free survival (RFS) was estimated by the Kaplan-Meier method, and statistical differences were tested by the log-rank test. RFS was measured from the date of surgery to the date of recurrence or the last follow-up. Deaths from other reasons were not defined as events for RFS.

Blood tests and physical examinations were done every

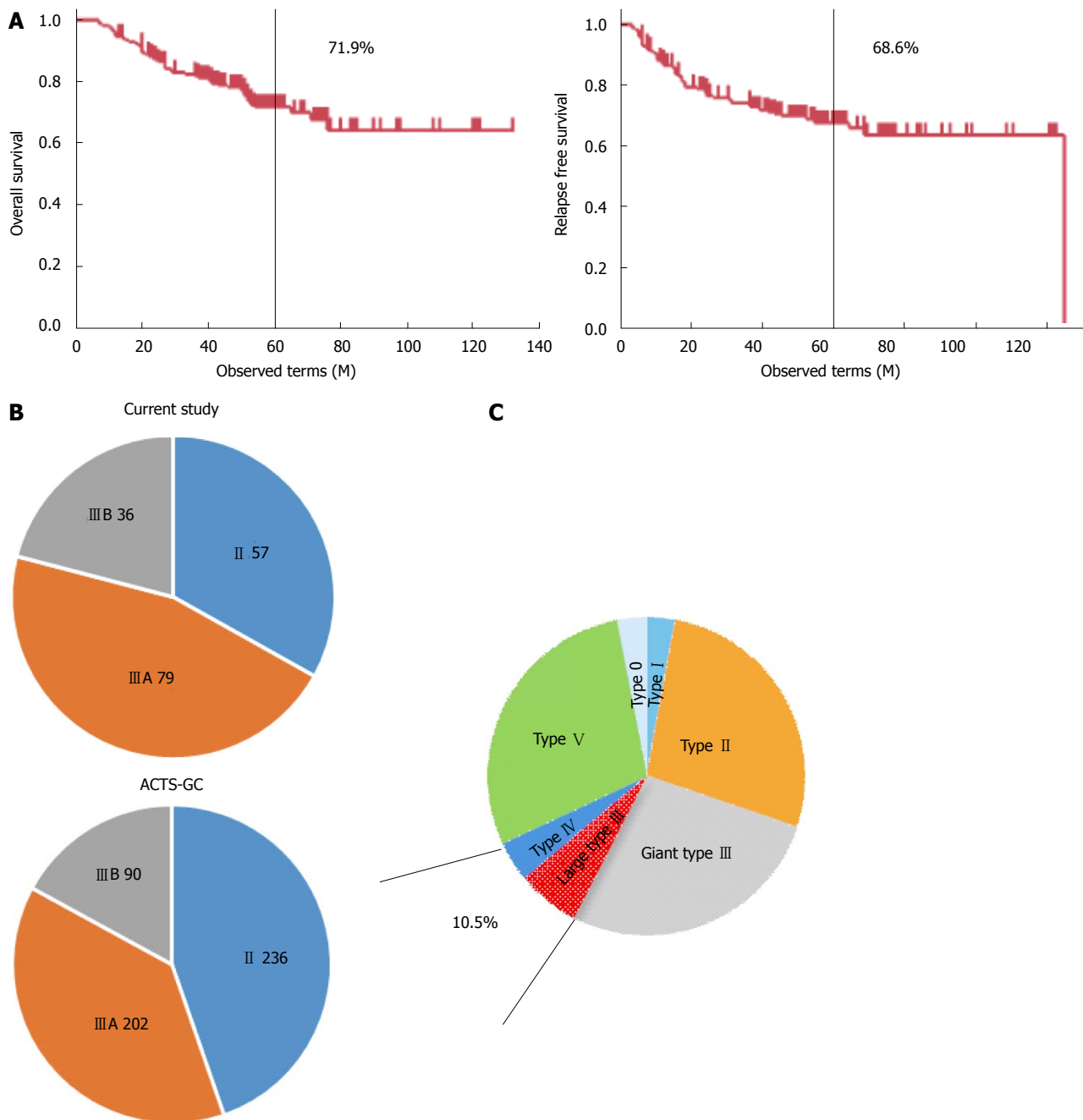


Figure 2 Prognosis of pathological stage II/III advanced gastric cancer patients who underwent curative gastrectomy followed by S1 postoperative adjuvant chemotherapy. A: Kaplan-Meier curves for overall survival (OS) (upper panel) and relapse free survival (RFS). Five year survival is shown; B: Stage distribution of pathological stage according to the 13th Japanese Gastric Cancer Association stage in Kitasato University in comparison with the ACTS-GC trial; C: Rate of each macroscopic feature in pathological stage II/III advanced gastric cancer. High-risk macroscopic features (type IV and giant type III) are seen in 10.5% as shown in this figure.

3 mo and imaging examinations were performed every 6 mo. Blood tests included a complete blood count and serum biochemistry including tumor markers such as CEA, CA19-9, and CA125. Diagnosis of recurrences was based on clinical reports of radiologists with reference to clinical findings (symptoms and blood test) or histological findings.

The median observation was 56 mo (range, 11 to 122 mo). Variables that had prognostic potential on univariate analysis ($P < 0.05$) were subjected to multivariate analysis with a Cox proportional hazards regression model. A value of $P < 0.05$ was considered significant. All statistical analyses were done with JMP, version 11 (SAS Institute, Cary, NC).

RESULTS

Prognosis of advanced gastric cancer patients with pathological stage II/III who underwent curative surgery followed by S1 postoperative adjuvant chemotherapy

The prognosis of gastric cancer patients with pathological stage II/III who underwent curative gastrectomy followed by S1 adjuvant chemotherapy was investigated first. Pathological stage II/III cases did not include those with pathological stage II T1 gastric cancer. Five-year OS and 5-year RFS were 71.9% and 68.6%, respectively (Figure 2A). These survival rates are almost the same as the survival outcomes in the ACTS-GC trial (71.7%

Table 1 Univariate prognostic analysis in pathological stage II/III advanced gastric cancer

Clinicopathological factors	Classification	Number	Univariate analysis (5-yr RFS)	Univariate analysis (<i>P</i> value)	5-yr OS	<i>P</i> value
Age	Young	74	77.40%	0.0082	82.30%	0.0024
	Elderly	98	56.90%		58.10%	
Sex	Male	120	62.50%	0.018	63.50%	0.0054
	Female	52	81.90%		83.10%	
Tumor location	Upper	54	59.60%	0.12	81.10%	0.027
	Middle	74	68.40%		76.10%	
	Lower	44	80.50%		59.20%	
Method	Total	100	67.40%	0.51	69.00%	0.18
	Distal	72	70.00%		76.00%	
Lymphadenectomy	D1	10	68.60%	0.95	56.00%	0.53
	D1+	16	64.30%		79.60%	
	D2	146	69.00%		72.20%	
Laparoscopic	Yes	25	77.30%	0.16	77.30%	0.2
	No	147	67.00%		70.90%	
Splenectomy	Yes	51	61.50%	0.2	65.80%	0.31
	No	121	71.50%		74.50%	
Transfusion	Yes	23	63.90%	0.58	68.00%	0.37
	No	149	69.30%		72.70%	
13 th JGCA pT	T2	65	80.30%	0.019	83.00%	0.021
	T3	105	61.90%		65.80%	
	T4	2	50.00%		50.00%	
13 th JGCA pN	N0	24	90.50%	0.0043	89.40%	0.022
	N1	82	74.00%		77.30%	
	N2	66	54.30%		59.10%	
13 th JGCA pStage	II	57	92.10%	< 0.0001	86.80%	< 0.0001
	III A	79	63.90%		76.00%	
	III B	36	43.00%		42.80%	
Lauren histology	Intestinal	60	63.60%	0.23	68.30%	0.27
	Diffuse	112	71.30%		74.20%	
INF	Alpha	13	76.90%	0.83	84.60%	0.53
	Beta	75	69.90%		77.50%	
	Gamma	84	66.40%		67.50%	
Lymphatic invasion	ly0	9	100.00%	0.2	100.00%	0.19
	ly1	45	74.80%		77.10%	
	ly2	62	63.70%		71.20%	
	ly3	56	63.90%		63.60%	
Vascular invasion	v0	16	87.10%	0.055	85.60%	0.043
	v1	55	69.90%		65.20%	
	v2	55	72.80%		83.50%	
	v3	46	55.70%		62.70%	
Macroscopic feature	High risk	18	35.70%	0.0003	34.00%	< 0.0001
	Average risk	154	72.60%		76.60%	

JGCA: Japanese Gastric Cancer Association.

and 65.4%)^[3]. On the other hand, the stage distribution included a lower rate of stage II gastric cancer and a higher rate of stage III gastric cancer than in the ACTS-GC trial (Figure 2B). These findings indicated that the patient population treated in our institute included more advanced gastric cancer than the ACTS-GC trial.

Classification of macroscopic features in pathological stage II/III advanced gastric cancer

Retrospective diagnosis with regard to the macroscopic features of gastric cancer was done by review of the recorded gastroscopic images in combination with the CT scan images (if primary tumors were visible on CT scan images, they were considered type I to IV macroscopic features, not type 0 macroscopic features). Among the type III macroscopic features, maximal tumor size was assessed by UGI series, and tumors with size of 8 cm or

beyond were defined as giant type III gastric cancers as previously described^[8]. As a result, high risk macroscopic features (type IV and giant type III) were identified in 18 cases (10.5%) (Figure 2C).

Multivariate Cox proportional hazards model for RFS identified macroscopic high risk as an independent prognostic factor in pathological stage II/III gastric cancer

RFS was compared with regard to various clinicopathological factors including macroscopic features (Table 1). There was a significant difference in RFS ($P = 0.0003$) between macroscopic high risk gastric cancer and average risk gastric cancer (Figure 3A). Five-year RFS of macroscopic high-risk gastric cancer was 35.7%, while that of average-risk gastric cancer was 72.6%. Other negative prognostic factors were older age ($P = 0.0082$),

Table 2 Multivariate Cox proportional hazards model in pathological stage II/III advanced gastric cancer

Clinicopathological factors	Classification	Number	Multivariate analysis for PFS (Hazard ratio)	Multivariate analysis for OS (95%CI)	P value	Hazard ratio	95%CI	P value
Age	Young	74	Reference		0.029	Reference		0.008
	Elderly	98	1.83	1.07-3.20		2.35	1.25-4.58	
Sex	Male	120	Reference	1.06-4.34	0.031	Reference	0.87-4.91	0.11
	Female	52	2.05			1.93		
Tumor location	Upper	54				2.84	1.24-7.19	0.013
	Middle	74				1.73	0.70-4.66	0.24
	Lower	44				Reference		
13 th JGCA pStage	II	57	Reference			Reference		
	III A	79	6.17	2.42-20.83	< 0.0001	2.25	0.93-6.27	0.08
	III B	36	8.48	3.11-29.70	< 0.0001	4.81	1.79-14.72	0.002
Vascular invasion	v0	16				Reference		
	v1	55				1.46	0.38-9.55	0.62
	v2	55				0.71	0.17-4.80	0.68
	v3	46				1.34	0.33-9.01	0.71
Macroscopic feature	High risk	18	2.29	1.06-4.63	0.036	2.74	1.17-6.15	0.021
	Average risk	154	Reference			Reference		

JGCA: Japanese Gastric Cancer Association.

male sex ($P = 0.018$), 13th JGCA pT ($P = 0.019$), 13th JGCA pN ($P = 0.0043$), and 13th JGCA stage ($P < 0.0001$). These significant prognostic factors for RFS excluding TNM factor components were applied to a multivariate Cox proportional hazards model, which identified the 13th JGCA stage ($P < 0.0001$), macroscopic high risk ($P = 0.036$), sex ($P = 0.031$), and age ($P = 0.029$) as independent prognostic factors as shown in Table 2. Kaplan-Meier survival curves are shown in terms of age (left panel of Figure 3B), sex (left panel of Figure 3C), and 13th JGCA stage (left panel of Figure 3D).

Multivariate Cox proportional hazards model for OS identified macroscopic high risk as an independent prognostic factor in pathological stage II/III gastric cancer

OS was compared with regard to various clinicopathological factors including macroscopic features (Table 1). There was significant difference in OS ($P < 0.0001$) between macroscopic high risk gastric cancer and average risk gastric cancer (Figure 3A). Five-year OS of macroscopic high risk gastric cancer was 34.0%, while that of average-risk gastric cancer was 76.6%. Other negative prognostic factors were older age ($P = 0.0024$), male sex ($P = 0.0054$), tumor location ($P = 0.027$), 13th JGCA pT ($P = 0.021$), 13th JGCA pN ($P = 0.022$), 13th JGCA stage ($P < 0.0001$), and vascular permeation ($P = 0.043$). These significant prognostic factors for OS excluding each TNM factor components were applied to the multivariate Cox proportional hazards model, which identified the 13th JGCA stage ($P = 0.0015$), macroscopic high risk ($P = 0.021$), age ($P = 0.0082$), and tumor location ($P = 0.013$) as independent prognostic factors as shown in Table 2. Each TNM factor was excluded, because these 3 factors are confounders for stage definition. Kaplan-Meier survival curves are shown in terms of age (right panel of Figure 3B), sex (right panel of Figure 3C), and 13th JGCA stage (right panel of Figure 3D).

Clinicopathological features of macroscopic high risk among pathological stage II/III gastric cancer patients who underwent standard treatment

Clinicopathological backgrounds with regard to the negative prognostic factors were then compared between the high-risk group and the average-risk group (Table 3). The macroscopic high-risk group included more patients with higher pathological T ($P = 0.0025$), and higher 13th JGCA pathological stage ($P = 0.0004$), while there were no significant differences in pN distribution and lymph node dissection level between the macroscopic high-risk group and the average-risk group. In our previous reports, lymph node dissection level was proven not to affect prognosis in these 172 cases^[11].

Recurrence patterns of macroscopic high risk gastric cancer

Recurrent cases were seen in 11 out of 18 cases with macroscopic high risk (Table 4). The 11 cases were composed of 7 giant type III gastric cancers and 4 type IV gastric cancers. Giant type III gastric cancer tended to have extra-regional lymph node recurrences, while type IV gastric cancer had peritoneal dissemination. We recently reported RTKs expression in gastric cancer, and HER3 and EGFR were of prognostic relevance in pathological stage II/III advanced gastric cancer^[12]. The expression patterns of RTKs such as EGFR, HER2, HER3, IGF1R and EphA2 are also included in Table 4 from the previous studies^[12]. Among the 11 recurrent cases, 9 showed strong expression (2+/3+) of EGFR, and 10 showed positive immunostaining (1+/2+) for HER3, which were both remnant independent prognostic factors in pathological stage II/III advanced gastric cancer^[12].

DISCUSSION

This study reported for the first time the outcomes of macroscopic high-risk gastric cancer (giant type III and

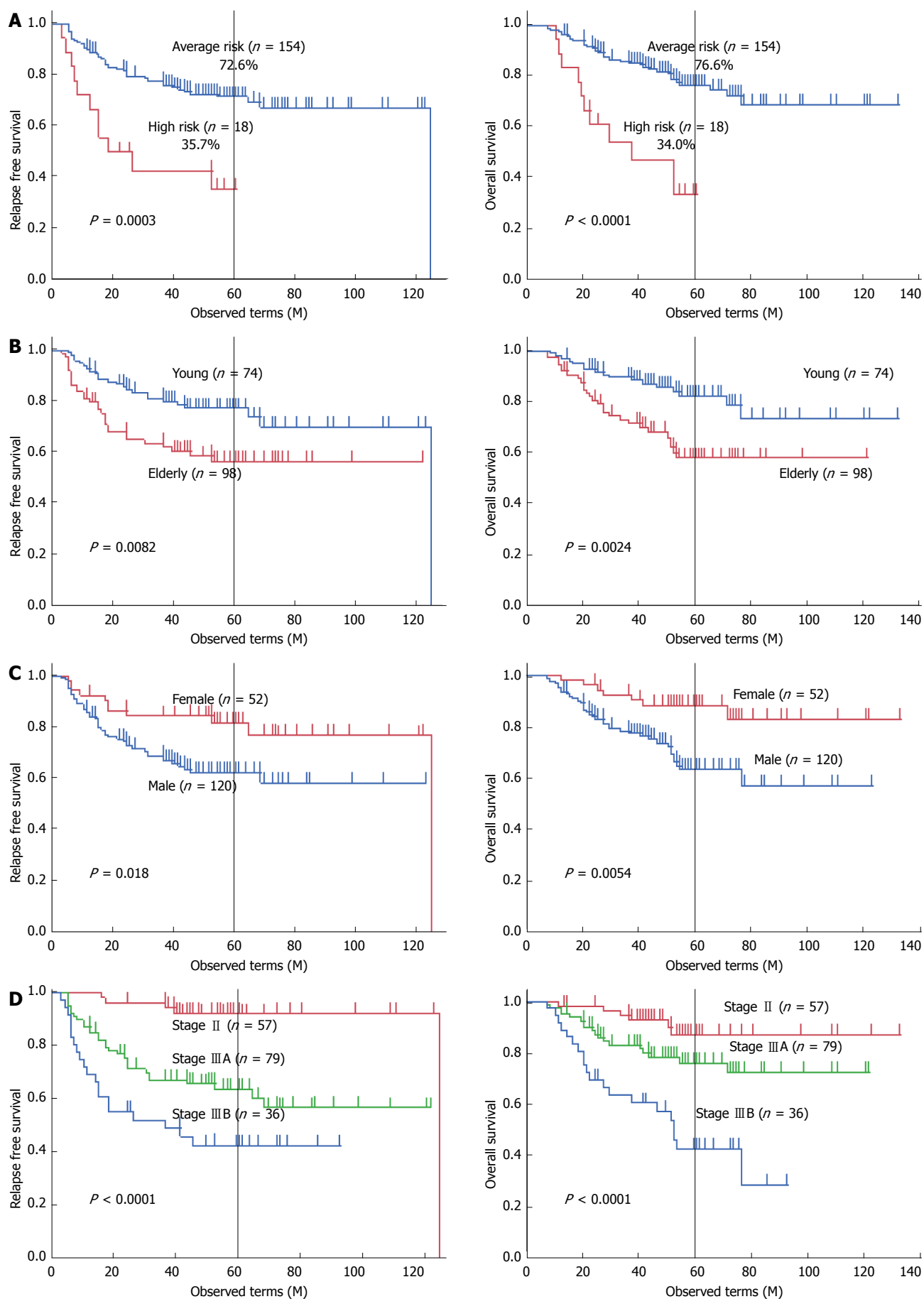


Figure 3 Survival curve of independent prognostic factors with regard to relapse free survival (left panel) and overall survival (right panel). A: Survival curve according to macroscopic features for the high-risk group and the average-risk group. Five-year survival is shown; B: Survival curve by age; C: Survival curve by sex; D: Survival curve by pathological stage according to the 13th JGCA stage. JGCA: Japanese Gastric Cancer Association.

Table 3 Relations of high risk macroscopic features to prognostic factors in pathological stage II/III advanced gastric cancer

Clinicopathological factors	Classification	Number	High risk gastric cancer <i>n</i> = 18	Average risk gastric cancer <i>n</i> = 154	<i>P</i> value
Age	Young	74	8	66	0.26
	Elderly	98	10	88	
Sex	Male	120	13	107	0.81
	Female	52	5	47	
Lymphadenectomy	D1	26	4	22	0.37
	D2	146	14	132	
Tumor location	Upper	54	3	51	0.32
	Middle	74	9	65	
	Lower	44	6	38	
13 th JGCA pT	T2	65	1	64	0.0025
	T3	105	17	88	
	T4	2	0	2	
13 th JGCA pN	N0	24	2	22	0.11
	N1	82	5	77	
	N2	66	11	55	
13 th JGCA pStage	II	57	3	54	0.0004
	III A	79	4	76	
	III B	36	11	25	
7 th UICC pT	T2	29	0	29	0.02
	T3	36	1	35	
	T4a	105	17	88	
7 th UICC pN	T4b	2	0	2	0.08
	N0	24	2	22	
	N1	45	1	44	
7 th UICC pStage	N2	40	4	36	< 0.0001
	N3	63	11	52	
	II A	13	0	13	
Vascular invasion	II B	37	2	35	0.94
	III A	46	2	44	
	III B	38	3	35	
	III C	38	11	27	
	v0	16	1	15	
	v1	55	6	49	
	v2	55	6	49	
	v3	46	5	41	

JGCA: Japanese Gastric Cancer Association.

type IV) treated by “local” standard therapy in Japan (or partly in some Asian countries) in stage II/III advanced gastric cancer. The ACTS-GC trial demonstrated that postoperative S1 chemotherapy could improve the prognosis of pathological stage II/III advanced gastric cancer^[2,3], but there has been no report on the prognosis of macroscopic high risk gastric cancer patients with pathological stage II/III who underwent standard treatment. In this study, 5-year RFS and OS of the macroscopic high-risk group were 35.7% and 34.0%, respectively, and the prognosis of gastric cancer patients with macroscopic high-risk was significantly poorer than that of those with average risk (72.6% and 76.6%, respectively). These results suggest that the present S1 postoperative chemotherapy is not sufficient to control such high risk disease, and novel therapeutic strategies are needed.

In the Western world, perioperative ECF chemotherapy has been shown to improve survival of gastric cancer patients when, ECF chemotherapy was compared to surgery alone^[10]. Gastric cancer with ECF chemotherapy showed 5-year OS of 36.3%, compared to 23.0% for surgery alone. This outcome is totally different from average-risk advanced gastric cancer in the Eastern

world, with an OS of 60%-70% of OS, whereas it is similar to gastric cancer with macroscopic high-risk. In the present cases, gastric cancer patients who were peritoneal cytology test-positive were excluded, because it represents stage IV in Japan, while the MAGIC trial may have included cytology test positive cases. In any case, the MAGIC trial demonstrated that potent preoperative chemotherapy has a great clinical potential in aggressive gastric cancer. In Japan, preoperative neoadjuvant chemotherapy was evaluated to validate the actual clinical effects including improvement of prognosis in very limited gastric cancer such as macroscopic high risk gastric cancer, namely giant type III and type IV gastric cancer^[9]; CS (cisplatin/S1) neoadjuvant chemotherapy was proposed as an effective regimens in gastric cancer with macroscopic high risk, and 5-year survival was recently reported to be around 30% in JCOG0210. This is inferior to our standard therapy results, likely because peritoneal cytology test negativity was not mandatory to register in JCOG0210.

Neoadjuvant therapy is a promising therapeutic strategy for giant type III and type IV gastric cancer. We have developed a docetaxel/cisplatin/S1 (DCS) chemotherapeutic regimen in metastatic gastric cancer^[16], and

Table 4 Initial recurrent sites and RTKs expression in high risk gastric cancer with relapse

Case	Age	Sex	13 th JGCA pT	13 th JGCA pN	13 th JGCA pStage	Macroscopic features	EGFR	HER2	HER3	IGF1R	EphA2	Initial recurrences
1	74	M	3	2	III B	Giant type III	2+	1+	2+	2+	1+	#16 LN
2	62	M	3	2	III B	Giant type III	2+	0+	1+	2+	0+	#20 LN
3	79	F	3	2	III B	Giant type III	2+	1+	1+	0+	1+	#13 LN
4	68	M	3	2	III B	Giant type III	3+	1+	1+	1+	0+	#16,13
5	68	M	3	2	III B	Giant type III	3+	0+	1+	1+	1+	#13 LN
6	45	M	3	2	III B	Giant type III	2+	3+	2+	0+	2+	#13 LN
7	69	M	3	2	III B	Giant type III	3+	3+	1+	1+	2+	liver
8	71	M	3	1	III A	Type IV	2+	0+	1+	1+	0+	#13 LN
9	69	F	3	1	III A	Type IV	2+	0+	2+	1+	0+	Peritoneum
10	69	M	3	1	III A	Type IV	1+	2+	0+	1+	1+	Peritoneum
11	59	F	3	2	III B	Type IV	1+	1+	1+	1+	0+	Peritoneum

JGCA: Japanese Gastric Cancer Association; M: Male; F: Female; EGFR: Epidermal growth factor receptor; HER2: Human epidermal growth factor receptor-2; IGF1R: Insulin-like growth factor 1.

KDOG1001 was developed to validate the clinical effect of DCS NAC in aggressive gastric cancer including giant type III and type IV. We are registering patients in this clinical phase II trial for such high-risk patients, and registration has almost been completed. DCS was recently compared to CS in neoadjuvant settings in high-risk gastric cancer with bulky N2 disease in JCOG1002, and detailed results of the clinical outcomes will be available soon. The first report of patients with high-risk advanced gastric cancer who underwent CS neoadjuvant chemotherapy should appear in April, 2017. Such potent chemotherapy would have a promising potential to improve the prognosis of aggressive gastric cancer.

Another therapeutic strategy we can propose in such aggressive gastric cancer is long-term postoperative adjuvant S1 chemotherapy^[17,18]. Gastric cancer that was cytology test-positive (CY1) or type IV showed a dismal prognosis, but detailed prognostic analysis showed that there were long-term survivors among the patients who underwent long-term postoperative adjuvant S1 chemotherapy. Okuyama *et al.*^[19] actually showed that 2-year administration of postoperative chemotherapy showed a better prognosis than 1-year administration in gastric cancer. This strategy might be very promising due to its easy feasibility, and should be considered as another therapeutic option. Giant type III and type IV gastric cancers are unique in their recurrence patterns, because minimal residual peritoneal disease is fundamental with regard to disease progression^[8,18]. This means that minimal residual disease of the peritoneum should be a primary therapeutic target. S1 is more effective against peritoneal disease than against other distant metastases such as liver metastases^[2] due to unknown mechanisms, thus, long-term S1 administration may be a reasonable rationale in macroscopic high-risk gastric cancer.

We previously identified HER3 immunostaining positive (defined as +1/+2 immunostaining) as an independent prognostic factor, and HER3 could be a promising therapeutic target^[12]. HER2 immunostaining (defined as +3 immunostaining) is the well-established molecular target in far advanced gastric cancer using trastuzumab^[20], but

HER2-positive cases are infrequently found in recurrent gastric cancer^[12]. Even in high-risk advanced gastric cancer, HER2-positive cases were infrequently seen (Table 4), while HER3-positive cases were frequently found. Moreover, EGFR-positive (defined as +2/+3) together with HER3-positive showed a dismal prognosis in advanced gastric cancer with pathological stage II/III^[12], and EGFR-positive together with HER3-positive was found in 9 of 11 recurrent cases among the high-risk advanced gastric cancer patients in this study. We are now investigating in vitro efficacy for tumor reduction by using cetuximab together with HER3 antibody. The combination treatments could have potential in the recurrent cases of high-risk gastric cancer.

The limitations of this study were that it was a single-center study, and the follow-up period was insufficient for definitive conclusions. Moreover, the sample size was small, especially for high-risk advanced gastric cancer. If this result is validated in a larger sample size in the near future, the conclusions would be strengthened. Moreover, this study only collected patients who underwent curative surgery plus adjuvant S1 chemotherapy, we didn't mention if these results can be seen from other patients with advanced gastric cancer.

In conclusion, this study demonstrated for the first time that macroscopic high-risk gastric cancer showed a poorer prognosis than average risk gastric cancer, and a novel therapeutic strategy should be urgently developed in order to improve outcomes in such cases in the near future.

COMMENTS

Background

High risk macroscopic appearance (giant type III and type IV) is known to show dismal prognosis in advanced gastric cancer, however it remains elusive whether it is true or not in advanced gastric cancer who underwent curative gastrectomy and the latest evidenced postoperative S1 adjuvant chemotherapy.

Research frontiers

This study investigated whether the high risk macroscopic appearance could be an independent prognostic factor in advanced gastric cancer who underwent

curative gastrectomy and postoperative adjuvant chemotherapy.

Innovations and breakthroughs

Macroscopic appearance can be preoperatively diagnosed, and it could be designated as a kind of preoperative surrogate marker for prognosis.

Applications

Macroscopic appearance is a good candidate for promising therapeutic strategy of neoadjuvant chemotherapy, the novel method in East Asia, if it is true.

Terminology

The size of the giant type III gastric cancer is defined as 8 cm or beyond in the preoperative imaging such as endoscopy, upper gastrointestinal series, and/or computed tomography.

Peer-review

Yamashita *et al* presented a study title as "Macroscopic appearance of Type IV and giant Type III is a high risk for a poor prognosis in pathological stage II /III advanced gastric cancer with postoperative adjuvant chemotherapy". The study has some new and interesting findings which authors believe they add some contribution to the literature. Authors were well summarized results, they have novel findings and discussion was pretty good.

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Prospective Study

Incidence of venous thromboembolism and the role of D-dimer as predictive marker in patients with advanced gastric cancer receiving chemotherapy: A prospective study

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Abstract

AIM

To investigate the incidence and risk factors of venous thromboembolism (VTE) in patients with advanced gastric cancer (AGC) receiving chemotherapy.

METHODS

All consecutive chemotherapy-naïve patients with AGC who would receive palliative chemotherapy between November 2009 and April 2012 in our hospital were recruited. Their pretreatment clinical and laboratory variables, including D-dimer, were recorded. The frequency of VTE development and survival rates during each chemotherapy cycle and regularly thereafter were assessed.

RESULTS

A total of 241 patients enrolled between November 2009

and April 2012 were analyzed. During a median follow-up duration of 10.8 mo (95%CI: 9.9-11.7), 27 patients developed VTE and the incidence of VTE was 17.5% (95%CI: 10.5-24.0, 12.0 events/100 person-years). The 6-mo and 1-year cumulative incidences were 7.8% (95%CI: 4.2%-11.4%) and 12.4% (95%CI: 7.3-17.2), respectively. Thirteen (48.1%) patients were symptomatic and the other 14 (51.9%) patients were asymptomatic. In multivariate analysis, pretreatment D-dimer level was the only marginally significant risk factor associated with VTE development (hazard ratio = 1.32; 95%CI: 1.00-1.75, $P = 0.051$).

CONCLUSION

The incidence of VTE is relatively high in patients with AGC receiving chemotherapy, and pretreatment D-dimer level might be a biomarker for risk stratification of VTE.

Key words: Advanced gastric cancer; D-dimer; Venous thromboembolism

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Core tip: The incidence of venous thromboembolism (VTE) is relatively high in patients with advanced gastric cancer receiving chemotherapy, and pretreatment D-dimer level is a risk factor for VTE. Considering the usefulness of D-dimer as a biomarker given its ease of use and low cost, pretreatment D-dimer might be a risk stratification factor for VTE development and patient selection for thromboprophylaxis.

Park K, Ryoo BY, Ryu MH, Park SR, Kang MJ, Kim JH, Han S, Kang YK. Incidence of venous thromboembolism and the role of D-dimer as predictive marker in patients with advanced gastric cancer receiving chemotherapy: A prospective study. *World J Gastrointest Oncol* 2017; 9(4): 176-183 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i4/176.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i4.176>

INTRODUCTION

In general, patients with cancer carry a significantly higher risk of venous thromboembolism (VTE) than case-control subjects without cancer^[1,2]. Gastrointestinal cancers including gastric cancer were ranked third in incidence of VTE, following hematological malignancies and lung cancer^[1]. Lee *et al*^[3] reported that patients with advanced gastric cancer (AGC) had a much higher likelihood of developing VTE (24.4%) than patients with lower-stage gastric cancer (0.5%-3.5%), and Blom *et al*^[1] observed that chemotherapy increased the VTE risk 2.2-fold. These findings suggest that VTE might be more common in patients with AGC receiving chemotherapy, who have several potential risk factors of VTE development including cancer, especially highly vulnerable gastric cancer, advanced stage, and chemotherapy. On the other hand,

patients with cancer who develop VTE also have shorter survival durations than those who do not develop VTE^[4-6]. Activation of hemostasis, as indicated by development of VTE reflects more aggressive tumor biology^[7], additionally, VTE development might hinder the continuation of chemotherapy, resulting in poor outcomes. Considering the relatively high incidence of VTE and its impact on survival, information about the VTE is important in patients with AGC receiving chemotherapy. While, the information about VTE in this cohort is not yet clear. Because most of the previous results were retrospectively analyzed with heterogeneous population and included this cohort as a part of small fraction among heterogeneous various groups^[8-11].

It has been challenging for oncologists to conduct a practical use of thromboprophylaxis effectively to prevent VTE. The major problem is that the increased rates of complications such as bleeding outweigh its efficacy^[12]. Thus, we must more precisely target thromboprophylaxis, especially in gastric cancer, since it has a high risk of bleeding at the endothelial lesion. Therefore, we need to confirm the exact incidence and identify the predictive factors of future VTE in this cohort. To address these issues, we conducted this prospective cohort study to determine the exact incidence and risk factors of VTE in patients with AGC who are undergoing palliative chemotherapy and assess whether VTE development correlates with survival.

MATERIALS AND METHODS

Study design and patients

This is a prospective observational single-center study. The cohort consisted of all consecutive patients with histologically confirmed adenocarcinoma of the stomach or esophagogastric junction that was in an advanced state (e.g., initially metastatic, locally inoperable, or recurrent), and who started palliative chemotherapy between November 2009 and April 2012 at Asan Medical Center, South Korea. All patients were chemotherapy-naïve or had undergone adjuvant chemotherapy 6 mo before being included in the study. All patients had adequate bone marrow, renal, hepatic, and cardiac functions that would allow them to withstand chemotherapy. The following patients were excluded: Those who presented initially with brain metastasis, had been treated with warfarin or low-molecular-weight heparin 4 wk before being screened for inclusion in the study, had undergone major surgery or experienced significant traumatic injuries 4 wk before screening, or were lost to follow up during the first two weeks without evidence of disease progression or VTE. All included patients were observed prospectively for at least 1 year after enrollment of the last patient or until death, loss of follow-up, or withdrawal of consent occurred. The study was approved by the institutional review board of Asan Medical Center and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines (ClinicalTrials.gov identifier: NCT01047618).

All participants provided written informed consent before enrollment.

Treatment and work-up

The chemotherapy regimens mainly included fluoropyrimidine plus platinum-based for the 1st-line, taxane-based for the 2nd-line, and irinotecan-based for the 3rd-line chemotherapy. Each chemotherapy line was continued until disease progression, intolerable toxicity, patient demand, or the attending physician's decision. Routine erythropoiesis-stimulating agent was not applied during the study period.

Within one week before starting palliative chemotherapy, we checked the clinicopathologic factors and laboratory tests including D-dimer. Close history taking, a physical examination, CBC, and chemistry analyses were performed at every chemotherapy cycle and regularly during the follow-up period. The response of each patient to the treatment was assessed every 6 wk. When VTE was suspected due to a constellation of new clinical symptoms or signs, imaging analyses were performed. Doppler sonography and/or CT venography were used to detect DVT, while chest CT and/or pulmonary artery CT angiography were employed to detect pulmonary embolism (PE). Incidentally detected VTE was also counted as an event.

Statistical analysis

The primary endpoint of this study was the incidence of VTE in patients with AGC receiving chemotherapy, and the secondary endpoints were to identify risk factors of VTE and to evaluate the impact of VTE on survival in this cohort. For risk factor analysis of VTE, we used time to VTE to discriminate early vs delayed development considering the different clinical significance. The baseline characteristics such as baseline biomarkers levels, clinical parameter, cancer diagnosis information, medication and therapy are expressed as the median value with a range (continuous variables) or frequency with proportion (%) (categorical variables). The incidence of VTE was calculated as both cumulative incidence and person-time (events/100 person-years). The statistical significance of the difference in characteristic between symptomatic and incidental VTE was assessed using the χ^2 test or Fisher's exact test. The time to VTE or overall survival (OS) were measured from the date of first chemotherapy to the date of diagnosis of VTE or death or were censored at the last follow-up date. The time to event data were summarized using the Kaplan-Meier method. Uni- and multivariate Cox regression models were used to detect the association between clinical or pretreatment factors and time to VTE or OS. In the multivariate regression model, all potential factors with a *P* value ≤ 0.15 on univariate analyses were employed to detect adjustment factors. The final models were determined by backward elimination. In the regression modeling, log-transformation for severely skewed variables was used to obtain more stable regression coefficients. All statistical analyses were

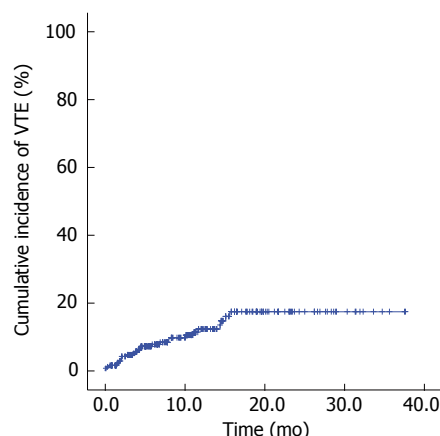


Figure 1 Cumulative incidence of venous thromboembolism. VTE: Venous thromboembolism.

performed by using SPSS software, version 21.0 (SPSS Inc., Chicago, IL, United States) and R software 2.10.1. All reported *P* values are two-sided and *P* values < 0.05 are considered to be statistically significant.

RESULTS

Patient characteristics and anticancer treatment

Between November 2009 and April 2012, a total of 261 consecutive patients were enrolled in this study. Of these patients, 20 (7.7%) did not receive chemotherapy or were lost to follow-up during the first 2 wk without evidence of disease progression or DVT, so they were excluded from the analysis. The remaining 241 patients had a median age of 56 years (range, 24-83 years) and 169 patients (70.1%) were male. The pretreatment clinicopathological and laboratory characteristics of all patients and those who developed VTE during palliative chemotherapy are summarized in Table 1. All patients received one or more cycles of chemotherapy; the median numbers of 9 cycles (range, 1-42) and 2 lines (range, 1-4).

Frequency and treatment of VTE

During a median follow-up duration of 10.8 mo (95%CI: 9.9-11.7), 27 patients developed VTE and the VTE incidence was 17.5% (95%CI: 10.5%-24.0%; 12.0 events/100 person-years). The 6-mo and 1-year cumulative incidences of VTE were 7.8% (95%CI: 4.2%-11.4%) and 12.4% (95%CI: 7.3-17.2%), respectively (Figure 1). Regarding VTE type, 19 of 27 patients (70.4%) developed deep vein thrombosis (DVT) only, 4 patients (14.8%) developed PE only, and 4 patients (14.8%) had both DVT and PE. The median time to VTE in these patients who developed VTE was 3.9 mo (95%CI: 2.8-5.1). VTE was detected within 3 mo in 10 patients (37.0%), from 3 to 6 mo in 6 patients (22.2%), from 6 mo to 1 year in 7 patients (25.9%), and after 1 year in the remaining 4 patients (14.8%). Thirteen patients (48.1%) had symptomatic VTE and the other 14 (51.9%) had incidentally detected VTE. A total of 25 patients received treatments for VTE, 22 took warfarin or

Table 1 Patients' pretreatment characteristics and risk of venous thromboembolism on univariate analysis

Variable	Total patients	Patients with VTE	Risk of time to VTE		
			HR	95%CI	P value
Total, n (%)	241 (100.0)	27 (11.2)			
Age (yr)					
< 65	178 (73.9)	18 (10.1)			
≥ 65	63 (26.1)	9 (14.3)	1.632	0.732-3.641	0.231
Gender					
Male	169 (70.1)	18 (10.7)			
Female	72 (29.9)	9 (12.5)	1.052	0.472-2.345	0.9
ECOG PS					
0-1	229 (95.0%)	27 (11.8)			
2	12 (5.0%)	0	0.046	0.000-99.991	0.432
BMI					
< 25	202 (83.8)	23 (11.4)			
≥ 25	39 (16.2)	4 (10.3)	0.793	0.274-2.296	0.669
Previous CVC					
No	233 (96.7)	25 (10.7)			
Yes	8 (3.3)	2 (25.0)	3.13	0.738-13.266	0.122
Previous gastrectomy					
No	209 (86.7)	26 (12.4)			
Yes	32 (13.3)	1 (3.1)	0.196	0.027-1.448	0.11
Primary tumor site					
Antrum/pylorus	94 (39.0)	10 (10.6)			
Body	32 (13.3)	9 (11.0)	1.131	0.458-2.791	0.79
Cardia/fundus	28 (11.6)	3 (10.7)	1.138	0.313-4.144	0.844
Diffuse	25 (10.4)	5 (20.0)	2.413	0.823-7.073	0.108
Histology					
W/D or M/D	84 (34.9)	6 (7.1)			
P/D or SRC	154 (63.9)	21 (13.6)	2.084	0.840-5.166	0.113
Unclassified	3 (1.2)	0	NA		
Peritoneal seeding					
No	137 (56.8)	11 (8.0)			
Yes	104 (43.2)	16 (15.4)	1.945	0.902-4.191	0.09
Liver metastasis					
No	156 (64.7)	20 (12.8)			
Yes	85 (35.3)	7 (8.2)	0.741	0.313-1.754	0.495
Lung metastasis					
No	220 (91.3)	26 (11.8)			
Yes	21 (8.7)	1 (4.8)	0.509	0.069-3.764	0.509
Bone metastasis					
No	225 (93.4)	24 (10.7)			
Yes	16 (6.6)	3 (18.8)	2.344	0.701-7.835	0.167
Number of metastatic sites					
0-1	109 (45.2)	9 (8.3)			
≥ 2	132 (54.8)	18 (13.6)	1.898	0.851-1.898	0.118
CEA (log) median (range, ng/mL)	2.5 (0.3-8070)		1.133	0.947-1.355	0.173
CA19-9 (log) median (range, U/mL)	19.3 (1.4-30800)		0.882	0.726-1.073	0.209
CA72-4 (log) median (range, U/mL)	5.1 (1.7-6490)		1.099	0.885-1.364	0.395
Hb median (range, g/dL)	12.0 (7.0-17.6)		0.956	0.786-1.163	0.653
WBC median (range, × 10 ⁹ /L)	7100 (2600-19300)		1	1.000-1.000	0.026
Platelet median (range, × 10 ⁹ /L)	274 (107-731)		1.001	0.998-1.005	0.508
CRP median (range, mg/dL)	2.01 (0.10-19.22)		1.056	0.952-1.053	0.302
Fibrinogen (log) median (range, × 10 ³ /L)	360 (66-897)		1.132	0.337-3.796	0.841
PAI-1 (log) median (range, × 10 ⁹ /L)	35.0 (2.0-112.0)		1.197	0.662-2.165	0.551
D-dimer (log) median (range, × 10 ⁹ /L)	1.02 (0.06-82.3)		1.401	1.069-1.836	0.015

BMI: Body mass index; CI: Confidence interval; CRP: C-reactive protein; CVC: Central venous catheter; log: Log-transformation; M/D: Moderate differentiation; P/D: Poor differentiation; SRC: Signet ring cell; VTE: Venous thromboembolism; W/D: Well-differentiated.

low-molecular-weight heparin, and the other three were treated with an inferior vena cava filter. The remaining two patients were treated conservatively. VTE-associated delay or discontinuation of chemotherapy occurred in 10 patients and was significantly more common in patients with symptomatic VTE than in patients with incidental VTE ($P =$

0.018). There were no cases of VTE-induced death (Table 2).

Risk factors of VTE

The pretreatment characteristics and laboratory data were assessed to determine the association with time to VTE development. In univariate analyses, log-transformed

Table 2 Clinical features of venous thromboembolism in advanced gastric cancer patients receiving chemotherapy *n* (%)

	Total (<i>n</i> = 27)	Symptomatic VTE (<i>n</i> = 13)	Incidental VTE (<i>n</i> = 14)	<i>P</i> value
Time to VTE duration (median, mo)	6.1	7.5	4.7	0.16
VTE incidence				
< 3	10 (37.0)	4 (30.8)	6 (42.9)	0.68
3-6	6 (22.2)	3 (23.1)	3 (21.5)	
6-12	7 (25.9)	3 (23.1)	4 (28.6)	
> 12	4 (14.8)	3 (23.1)	1 (7.2)	
Types of VTE				
DVT	19 (70.4)	8 (61.5)	11 (78.6)	0.608
PTE	4 (14.8)	3 (23.1)	1 (7.2)	
PTE + DVT	4 (14.8)	2 (15.4)	2 (14.3)	
Treatment of VTE				
Medication (anticoagulation)	22 (81.4)	10 (76.9)	12 (75.7)	0.031
IVC filter	3 (11.1)	3 (23.1)	0	
No treatment	2 (7.5)	0	2 (14.3)	
Delay of chemotherapy				
None	17 (62.9)	5 (38.5)	12 (85.7)	0.018
Yes	10 (37.1)	8 (61.5)	2 (14.3)	

DVT: Deep vein thrombosis; PTE: Pulmonary thrombosis; VTE: Venous thromboembolism; IVC: Inferior vena cava.

Table 3 Multivariate analysis of risk factors for venous thromboembolism¹

Variable	HR	95%CI	<i>P</i> value
Prior gastrectomy (no <i>vs</i> yes)	0.25	0.03-1.89	0.178
History of CVC (no <i>vs</i> yes)	2.21	0.51-9.50	0.286
D-dimer (log)	1.32	1.00-1.75	0.051

¹All potential prognostic factors with a *P* value ≤ 0.15 on univariate analyses were entered into the multivariate Cox models. The final models were determined by backward elimination.

D-dimer levels and WBC count had a statistically significant association with time to VTE (Table 1). Prior history of central venous catheter, differentiation, prior history of gastrectomy, peritoneal seeding, and number of metastatic sites trended toward a potential risk factor for VTE (*P* ≤ 0.15). In multivariate analysis, any prognostic factors were not statistically significant, but the log transformed D-dimer level was only a marginally significant risk factor in the final model (HR = 1.32, 95%CI: 1.00-1.75, *P* = 0.051) (Table 3).

In case of patients with VTE, the mean levels of D-dimer were 4.19 × 10⁹/L (baseline) and 11.18 × 10⁹/L (at the time of VTE development), respectively, with a significant increase of D-dimer levels at the time of VTE development (*P* = 0.004). Additionally, according to symptom development of VTE, D-dimer levels were increased significantly in patients with symptomatic VTE (*P* = 0.004), on the other hand, those in patients with incidental VTE showed only numerical increase (*P* = 0.198) (Table 4).

There was no significant difference in the time to VTE according to fluoropyrimidine or different platinum use as the 1st-line treatment or the addition of targeted agents or angiogenesis inhibitors (Table 5).

VTE as a prognostic factor

During a median observational duration of 13.8 mo

Table 4 Comparisons of D-dimer levels between baseline and time of venous thromboembolism development

	Total (<i>n</i> = 27)	Symptomatic VTE (<i>n</i> = 13)	Incidental VTE (<i>n</i> = 14)	<i>P</i> value
Baseline D-dimer	4.19	3.62	4.72	0.835
D-dimer at the time of VTE development	11.18	14.11	8.45	0.436
	<i>P</i> value	<i>P</i> value	<i>P</i> value	
	0.004	0.01	0.198	

VTE: Venous thromboembolism.

(95%CI: 11.6-14.8), the median OS was 14.2 mo (95%CI: 11.8-16.6). There was no significant difference in OS between patients with and those without VTE (13.8 mo, 95%CI: 9.3-18.3; 14.2 mo, 95%CI: 11.7-16.7, *P* = 0.559) (Figure 2). According to symptom development of VTE, patients with symptomatic VTE was not also noted statistically significant difference in OS compared with patients without VTE (*P* = 0.337). According to VTE type, there was no significant difference in OS between patients with DVT alone and with PE alone or PE/DVT (*P* = 0.597). In the analysis that excluded four patients who developed VTE after 12 mo, there was no significant difference in OS between patients with or without VTE (*P* = 0.609). On the contrary, in the analysis that excluded 10 patients who developed VTE before 3 mo, there was no significant difference in OS between patients with or without VTE (*P* = 0.337).

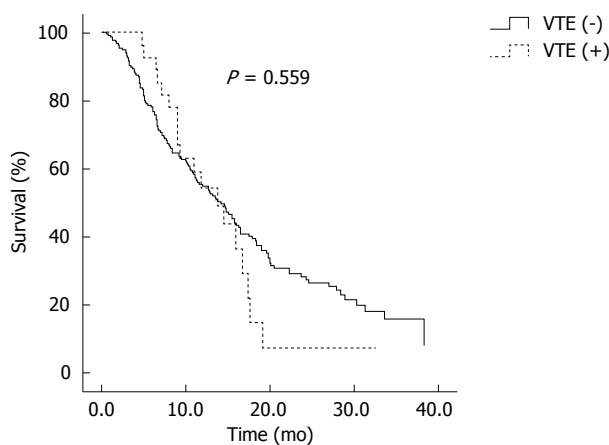
DISCUSSION

The current study assessed the incidence and risk factors of VTE as well as the influence of VTE on survival in AGC patients undergoing palliative chemotherapy. To our knowledge, this is the first prospective study to evaluate VTE in a homogeneous cohort. This study showed a relatively high cumulative incidence of VTE and the role

Table 5 Time to venous thromboembolism according to chemotherapeutic agent used for 1st-line chemotherapy

Variable	Patients (n)	Patients with VTE n (%)	Risk of time to VTE		
			HR	95%CI	P value
Fluoropyrimidine					
SP	96	11 (11.5)			
XP	22	3 (13.6)	1.67	0.47-6.18	0.422
Platinum					
XP	22	3 (13.6)			
XELOX	27	4 (14.8)	0.94	0.21-4.25	0.94
Addition of targeted agents					
Conventional chemotherapy	158	17 (10.8)			
+ targeted agents	83	10 (12.0)	1.21	0.56-2.65	0.627
Addition of VEGFR inhibitors					
Conventional chemotherapy	217	27 (12.4)			
+ VEGFR inhibitors	24	0	0.04	0.00-12.13	0.227

SP: TS-1 + cisplatin; XP: Capecitabine + cisplatin; XELOX: Capecitabine + oxaliplatin, targeted agents, Vorinostat, sorafenib, Bevacizumab, or trastuzumab +/- pertuzumab; VEGFR: Inhibitors, sorafenib or bevacizumab; VTE: Venous thromboembolism.

**Figure 2** Overall survival according to the development of venous thromboembolism. VTE: Venous thromboembolism.

of pretreatment D-dimer as a risk factor for development of VTE. Although the development of VTE was not correlated with poor survival, patients with symptomatic VTE had more interruptions or delays of chemotherapy than those with incidental VTE.

The 6-mo, 1-year, and 2-year cumulative incidences of VTE were 7.8%, 12.3%, and 17.3%, respectively. Our results showed a relatively high incidence of VTE despite AGC being a representative frequent site in cancer-associated VTE^[13]. In our previous report of retrospective analysis of a similar patient population, the 1- and 2-year cumulative incidences of venous VTE were 3.5% and 4.9%, respectively^[8]. Other prior studies also reported VTE incidences of 5.3%-11.4%^[14-16], which was somewhat lower than our results. The reason for this discrepancy should be preferentially presumed that this study was conducted for homogeneous patients who were high tumor burden of advanced state and receiving palliative chemotherapy. Regarding tumor burden in cancer-associated VTE, Lee *et al.*^[3] reported that the VTE incidence increased with stage in gastric cancer patients. Considering that stage is correlated with

tumor burden in cancer patients, we can give careful consideration to our results that a history of previous gastrectomy and multiple metastases were slightly related with the development of VTE, but the correlation was not statistically significant. Regarding chemotherapy of cancer-associated VTE, prior studies reported that chemotherapy is another important risk factor for VTE development in cancer patients^[1,17]. In the current study, 13 (46.4%) and 20 (71.4%) patients developed VTE within 3 and 6 mo, respectively, which suggests that starting chemotherapy is an important risk factor for VTE development. Blom *et al.*^[1] also reported that the risk of thrombosis was the highest in the first 3 mo after the diagnosis of cancer and declined thereafter, which supports the finding of the current study.

In multivariate analysis, pretreatment D-dimer level was the only marginally significant risk factor for VTE development. D-dimer, a general biomarker that globally indicates hemostasis and fibrinolysis activation, is a well-studied biomarker in the diagnosis and management of VTE in non-cancer patients^[18]. Khorana *et al.*^[17] proposed a predictive model for chemotherapy-associated VTE that included the primary cancer site, pretreatment platelet count, hemoglobin, leukocyte count, and body mass index. However, D-dimer level was not included in this model, which might be because the pretreatment D-dimer level was not routinely checked in the target population. In the subsequent studies, Ay *et al.*^[19] reported that a high D-dimer level predicted occurrence of VTE in a variety of cancer patients, and Arpaia *et al.*^[20] demonstrated that pretreatment D-dimer was correlated with chemotherapy-associated VTE. However, these studies only included a small number of gastric cancer patients, 35 of 821 (4.3%) and 10 of 124 (8.1%). Moreover, subcohorts of these patients were heterogeneous since the patients were treated variably with surgery or radiotherapy (or even untreated). Thus, the role of D-dimer as a risk factor of VTE in AGC patients receiving chemotherapy remains to be clarified. We demonstrated here that pretreatment D-dimer was indeed an independent risk factor of VTE in

this homogeneous cohort. This means that pretreatment D-dimer might be used to more precisely target patients for thromboprophylaxis and lead to enhanced use of thromboprophylaxis in AGC patients treated with palliative chemotherapy. On the other hand, prior established risk factors including the aforementioned Khorana score were not associated with VTE in this study (data not shown). A possible explanation might be the small sample size; however, the unique characteristics of stomach cancer or even ethical characteristics might also contribute.

The occurrence of VTE has been reported to have a significant adverse effect on survival in many studies^[3,5,6]. However, we found no statistical difference in survival according to VTE development. The results did not change after adjustment for the presence of symptoms and signs, VTE type, or detection time. The reason for the negative result in the current study may be too small a sample size to detect a difference. On the other hand, due to the short survival of patients with advanced-stage disease, VTE may have a greatly reduced impact on survival. Previous studies reported that an impact of VTE on survival might be somewhat different from that in our study because they mainly included localized stage of cancer^[3] or showed a prominent impact of VTE on survival in subgroups of patients with localized cancer^[5] or in those with breast cancer, which has a characteristic longer survival duration^[6]. Our previous retrospective study with a larger cohort of similar patients also reported that VTE was not a statistically significant factor for survival^[8]. As such, whether VTE really has adverse effect on survival in this cohort should be further clarified. Meanwhile, more patients with symptomatic VTE than asymptomatic VTE had chemotherapy interruptions or delays. Although symptomatic VTE did not show poor survival in the subgroup analysis, we should make an effort to detect VTE before symptoms develop.

This study has several limitations. First, we did not evaluate serial measurements of D-dimer; thus, we could not identify the usefulness of the serial changes as a predictive marker for VTE. Considering that the current study showed that D-dimer level at the time of VTE development is significantly increased compared with that at baseline in patients with VTE, serial measurements of D-dimer might detect early changes and predict the development of VTE. Another issue is that the current study did not calculate the proper number of patients, so it might not have been adequately powered to detect statistically significant differences in other characteristics such as risk factors or survival. For these reasons, it is obvious that the present study might not be a confirmative, rather preliminary study for hypothesis generation. Despite these limitations, the current study showed the incidence of VTE and role of pretreatment D-dimer as risk factors in a homogeneous group of AGC patients receiving palliative chemotherapy. Considering the usefulness of D-dimer as a biomarker such as its ease of use and low cost, pretreatment D-dimer might be used as a risk stratification factor for VTE and in patient selection for thromboprophylaxis.

In conclusion, the incidence of VTE is relatively high in patients with AGC receiving chemotherapy, and pretreatment D-dimer level is a risk factor for VTE. Considering the usefulness of D-dimer as a biomarker given its ease of use and low cost, pretreatment D-dimer might be a risk stratification factor for VTE development and patient selection for thromboprophylaxis.

COMMENTS

Background

Patients with cancer, especially gastric cancer, are higher risk of venous thromboembolism (VTE). Among cancer, advanced status such as recurrent, metastatic, or locally advanced, is high risk factor and treatment with chemotherapy is also risk factor of VTE. So, information about the VTE in patients with advanced gastric cancer (AGC) receiving chemotherapy is important. However, there has been no report about this homogeneous group, so they planned this study.

Research frontiers

This study is the first prospective study focused to evaluate VTE and risk factor of VTE in this homogeneous cohort. They reported an incidence of VTE and role of pretreatment D-dimer as risk factor.

Innovations and breakthroughs

The incidence of VTE is relatively high in patients with AGC receiving chemotherapy 17.5% (95%CI: 10.5%-24.0%; 12.0 events/100 person-years), and pretreatment D-dimer level is a risk factor for VTE.

Applications

Considering the usefulness of D-dimer as a biomarker given its ease of use and low cost, pretreatment D-dimer might be a risk stratification factor for VTE development and patient selection for thromboprophylaxis.

Terminology

AGC: Gastric cancer of advanced status such as initially metastatic, locally inoperable, or recurrent.

Peer-review

Naturally an increased level of circulating fibrin d-dimer may be a good parameter to control the evolution of gastric cancer venous originated metastasis.

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***Helicobacter pylori* recurrence after eradication in Latin America: Implications for gastric cancer prevention**

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Abstract

AIM

To estimate *Helicobacter pylori* (*H. pylori*) recurrence rate in Latin America, a region with a significant *H. pylori* prevalence and gastric cancer burden.

METHODS

PubMed, LILACS, SciELO, Cochrane databases and abstracts from relevant meetings were reviewed. Information collected included: Participants' characteristics, recruitment strategy, diagnostic modality, treatment arms, follow-up and recurrence rates. Recurrence was calculated using 100-patients-year rates, and data were pooled using a random effects model. The I^2 statistic assessed between study heterogeneity. Meta-regression analyses evaluated for effect modifying variables.

RESULTS

Literature search yielded 163 articles. Twelve studies involving 4848 patients from 9 countries met inclusion criteria. Four hundred and thirty-two reinfections were recorded in 5487 person-years of follow-up. Pooled analysis showed a recurrence rate of 7.9 cases per 100 person-years (95%CI: 5.3-10.5). Meta-regression revealed that neither the antibiotic schema, a second antibiotic course, nor the diagnostic modality had an impact on the observed risk of recurrence. The recurrence rate in the first year after treatment, predominantly recrudescence,

was 11.2 (6.1-16.4) per 100 patient years. Recurrence in subsequent years, was only 6.2 (3.8-8.7).

CONCLUSION

H. pylori recurrence rates in Latin America are significant, and with geographic variability, yet are acceptable based upon the current literature for consideration of large scale intervention trials. Further research in Latin America is warranted to evaluate the efficacy, cost-effectiveness, and potential adverse outcomes of proposed eradication programs.

Key words: Gastric cancer; Reinfection; Hispanic; *Helicobacter pylori*; Latin America

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Core tip: Latin America has a high burden of gastric cancer mortality, with significant geographical variability, which offers the opportunity for prevention trials and interventions. Recent trials and meta-analysis show that *Helicobacter pylori* (*H. pylori*) eradication reduces the risk of gastric adenocarcinoma. *H. pylori* reinfection rates in Latin America are similar to those seen in Asian trials. Recurrent cases occur mostly within the first year suggesting treatment failure (re-growth), not reinfection. These findings were not significantly modified by diagnostic modality, the antibiotics selected, retreatment, or the time check for eradication success. Eradication programs are a potentially attractive strategy for gastric cancer prevention in Latin America.

Corral JE, Mera R, Dye CW, Morgan DR. *Helicobacter pylori* recurrence after eradication in Latin America: Implications for gastric cancer prevention. *World J Gastrointest Oncol* 2017; 9(4): 184-193 Available from: URL: <http://www.wjgnet.com/1948-5204/full/v9/i4/184.htm> DOI: <http://dx.doi.org/10.4251/wjgo.v9.i4.184>

INTRODUCTION

Gastric cancer is the third most common cause of cancer mortality globally, and the leading infection-associated cancer^[1,2]. Of the 989000 gastric cancer cases in the world in 2008, 78% (770000) were estimated to be attributed to *Helicobacter pylori* (*H. pylori*) chronic infection^[3]. Gastric cancer has a marked geographic variability^[4,5]. Latin America has a particularly high burden of prevalent *H. pylori* infection and gastric cancer incidence and mortality^[6-8]. Estimated age-standardized mortality rates for males per 100000 are elevated in Honduras (22.3), Costa Rica (16.8), Peru (18.2), Chile (15.0), and Ecuador (20.7)^[5,9]. A concentration of incident gastric cancer is observed in the mountainous regions along the Pacific littoral, including in lower incidence countries (e.g., Mexico), which may offer the opportunity for focused prevention trials and interventions^[5].

Recent trials and a meta-analysis suggest that

screening and eradication of *H. pylori* can reduce the risk of gastric cancer^[10,11]. The Shangdong Intervention Trial, the largest randomized clinical trial to date, had a 53% *H. pylori* cumulative recurrence rate at 7 years, yet demonstrated a significant reduction in gastric cancer at 14.8 years (OR = 0.6, 95%CI: 0.4-0.9)^[10]. Trial participants were principally middle-aged Asian adults, and the generalizability of results to other populations is uncertain^[12]. Two subsequent meta-analyses confirmed the findings, while noting that the results were primarily driven by trials conducted in Asia^[11,13]. The International Agency for Cancer Research (IARC) has recently called for the design and study of large scale interventions for gastric cancer prevention in high incidence regions of the world, including Latin America^[12].

The *H. pylori* infection recurrence rate after eradication therapy is the critical determinant of the efficacy of an *H. pylori* eradication program designed to reduce the burden of gastric cancer. This review aims to estimate the reinfection rate of *H. pylori* after completion of antibiotic treatment in Latin America based upon existing literature. We present overall recurrence rates which includes both recrudescence (also called re-growth: Same strain, dominant in the first year after eradication) and reinfection (new strain, dominant in subsequent years), as the majority of studies do not genotype *H. pylori* strains.

MATERIALS AND METHODS

Review methods and reporting were performed according to the PRISMA guidelines^[14]. Literature databases PubMed (United States National Library of Medicine), LILACS (Latin America and the Caribbean Literature on Health Sciences), SciELO (Scientific Electronic Library Online) and Cochrane (the Cochrane Collaboration) were included as well as the abstracts from three major gastroenterology and infectious disease meetings [Digestive Disease Week (DDW), American College of Gastroenterology Scientific Meeting (ACG), and ID Week (IDW)]. Studies evaluating *H. pylori* reinfection in the 20 countries comprising Latin America, as defined by the United Nations Educational Scientific and Cultural Organization^[15], published in any language up to November 1st 2014 were included.

The search was performed in PubMed using the following sequence: *H. pylori* (MeSH term) AND [Recurrence (MeSH) or Recrudescence (MeSH) or Reinfection (not MeSH term)] AND (MeSH terms Latin America or Central America or South America or Argentina or Bolivia or Brazil or Colombia or Costa Rica or Cuba or Chile or Dominican Republic or Ecuador or El Salvador or Guatemala or Honduras or Mexico or Nicaragua or Panama or Paraguay or Peru or Puerto Rico or Uruguay or Venezuela). No other filters or limits were used. Analogous strategies were used to search the other two databases and the meetings' abstracts. Three additional meta-analyses relevant to the study were reviewed for further references^[16-18].

Information coding

Three investigators (Juan E Corral, Corey W Dye and

Douglas R Morgan) independently reviewed titles and abstracts for selection of potentially relevant articles. For journal manuscripts, full text articles were retrieved for further review. Titles that could not be associated with an abstract were excluded from review. A priori, studies with a sample smaller than 50 patient-years (PYs) and studies reporting same populations as other previously registered were excluded from meta-analysis. Citations of retrieved articles were reviewed for studies that may have been missed or were absent from our database queries. Authors were not contacted to provide additional information.

The following information was abstracted from each article: Year of publication, first author, country, information regarding participants (age, recruitment strategy), treatment arms (number of arms, medications used and duration in each arm), follow-up details (duration, intervals of appointment), diagnostic modality and recurrence rates. The interval of possible recurrence started with the last day of antibiotic regimen treatment, and ended with the day of follow-up *H. pylori* diagnostic testing; the last day of treatment was chosen to optimally account for eradication regimens of varying duration. In a given study, if there was more than one follow-up *H. pylori* diagnostic test for recurrence, each testing result was documented independently. The earliest time interval to consider infection recurrence and to be included in the review was 6 mo.

The quality of data (risk of bias) was assessed recording 5 variables, using the same methodology as Camargo *et al.*^[17] Antibiotic strategy was recorded in detail (medications and length of treatment) and was also scored as an ordinal variable [0 = only one antibiotic without a proton pump inhibitor (PPI), ranitidine or bismuth; 1 = either one antibiotic and a PPI or two antibiotics but no PPI (ranitidine or bismuth allowed); 2 = includes two antibiotics and a PPI (regardless of scheme, for example, triple, quadruple, sequential)].

Statistical analysis

All treatment arms in each study were reviewed individually. Cases were allocated in two groups: The patients that received antibiotics and those that received either placebo or an antacid medication (PPI, H₂ blocker or bismuth) but without antibiotics. Only antibiotic arms were included in meta-analysis. The number of patients with a negative test immediately after treatment (range 4–8 wk after antibiotic course) were recorded for the intention to eradicate analysis. The patients compliant with subsequent *H. pylori* testing were analyzed for our main analysis, and per our protocol, in this group, the patients lost to follow-up between eradication test (post antibiotics) and subsequent testing were not included. We also documented whether a second antibiotic course was offered for those patients with persistent infection or not.

We used a random-effects model to summarize recurrence rates. Summary reinfection rates and corresponding

95%CIs were calculated using the Poisson distribution. Forest plot graphs were created with 95%CIs. Given the relevance of differentiating between recrudescence (re-growth) and reinfection, subgroup analysis was performed for studies that looked for *H. pylori* recurrence at one year or less (< 53 wk cutoff) and those with longer follow-up^[16]. Pooled recurrence rates were calculated for different points in time for Latin America, starting at the first six months after completing antibiotics and for all subsequent years where data was available.

A secondary analysis was conducted with four additional comparisons: Recurrence 3 years after eradication, recurrence in studies enrolling children compared to studies restricted to adults, antibiotics regimens with high (> 75%) or low (≤ 75%) eradication success, and studies that assessed recurrence with endoscopy and biopsy compared to other diagnostic methods.

Meta-regression analyses were performed to evaluate for five effect modifying variables: Study population (community volunteers, patients with duodenal ulcers, dyspepsia, or intestinal metaplasia), *H. pylori* diagnostic modality (with or without urea breath test), quality of antibiotic treatment (0 to 2 points), possibility of a second antibiotic course, and length of follow-up (in years). Between-study heterogeneity was quantified using the *I*² statistic. Finally, publication bias was investigated by visual inspection of funnel plots. All statistical analyses were performed with Stata version SE 11.2 (Stata-Corp, College Station, TX, United States). The study plan and results were reviewed by a biomedical statistician (Robertino Mera).

RESULTS

The literature search resulted in 164 articles from the following sources: PubMed (104), LILACS (40), SciELO (20), and Cochrane (0) (Figure 1). Four abstracts were considered relevant from our review of conference reports (ACG 1, DDW 3, IDW 0). Two additional articles were identified after screening the references of manuscripts found in first review. After excluding 139 irrelevant or duplicate publications, 25 full text articles were retrieved for further evaluation, of which 7 were excluded because of incomplete data or duplicate samples and six additional per protocol for their small sample size (< 50 person-years of follow-up).

In summary, 12 studies from 9 countries met criteria for inclusion, which were published between 1991 and 2014 (Table 1). Ten studies included only adults, and an additional 2 studies included both adults and children. Eleven manuscripts were written in English and one in Spanish. Time to evaluate *H. pylori* eradication success ranged from 4 to 13 wk after last day of antibiotics (3 studies reported percentage of successful treatment “after randomization” without further details). Follow-up ranged from 6 mo to 16 years. These twelve studies encompassed 4848 patients [4685 patients received a treatment regimen that included antibiotics, 163 were

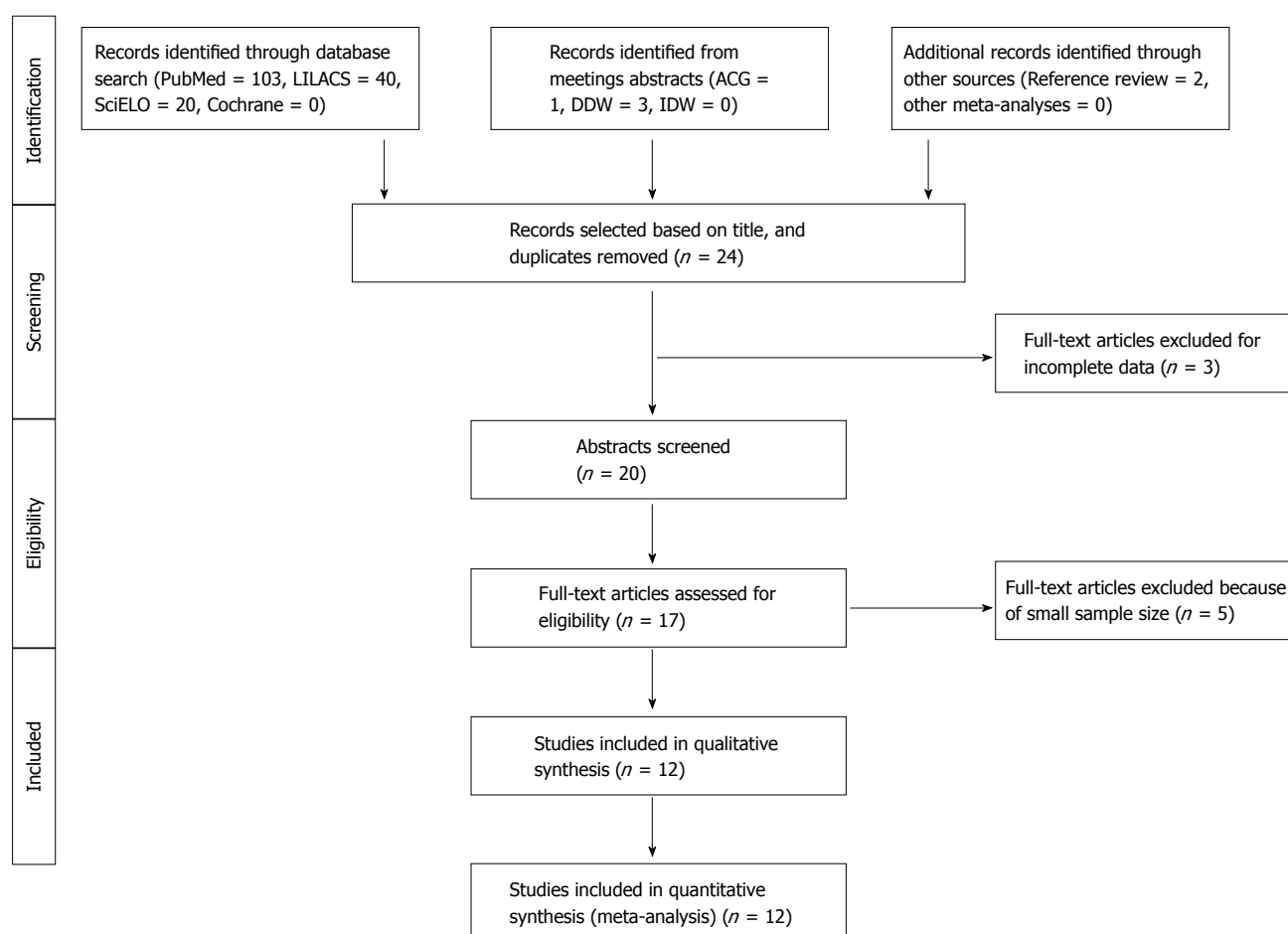


Figure 1 Latin America *Helicobacter pylori* recurrence: Study selection flow diagram (PRISMA 2009). DDW: Digestive Disease Week; ACG: American College of Gastroenterology Scientific Meeting; IDW: ID Week.

assigned to a placebo group or other treatment arm without antibiotics (only anti-acids)]. In these studies, the mean eradication rate after initial treatment was 72.2%, with a range of 30.2% to 100%. Reinfection rates ranged from 1.8% to 85.4%. In total, there were 432 reinfection events recorded in 5487 PYs follow-up in patients with sufficient data to calculate recurrence rates. Of the 4848 patients, 2172 (44.8%) did not complete follow-up diagnostic testing (Table 2).

Pooled analysis showed an overall recurrence rate of 7.9 cases per 100 PYs (95%CI: 5.3-10.5) (Figure 2). Analysis on an intention to eradicate basis (those with a negative test immediately after treatment) had a recurrence rate of 7.1 (4.7-9.6) per 100 PYs. The recurrence rate in the first year after treatment, postulated to be predominantly recrudescence, was 11.2 (6.1-16.4) per 100 PYs (all 12 studies included), while recurrence in subsequent years, an estimate of reinfection, was 6.2 (3.8-8.7) per 100 PYs. The cumulative reinfection rate at the 5 and 7 year time points were 36.2 and 48.6 per 100 PYs, respectively. Recurrence rates from different countries were combined for the first 6 years, 12 and 16 years (only one study had follow-up beyond 5 years^[19]). Recurrence rate were

higher in the first six months and decreased afterwards. Data from year 4 and 5 were combined as they had few PY follow-up (132 and 98, respectively). After the first year, reinfection rates ranged from 3.4 per 100 PYs in year 2 to 6.3 per 100 PYs in the combined 4-5 year period (Figure 3).

In a secondary analysis, the reinfection rate was lower when using a 3-year time cutoff; with an estimated rate of 3.8 (95%CI: 1.6-6.1) cases per 100 PYs. Recurrence rates were two times higher in studies that enrolled children compared to those that only enrolled adults 12.3 (95%CI: 9.6-14.9) vs 6.9 (95%CI: 4.2-9.6) cases per 100 PYs. There was no significant difference in recurrence rates among trials with high or low initial eradication success [7.8 (95%CI: 3.4-12.3) vs 8.4 (95%CI: 4.6-12.1), respectively]. Recurrence rates were higher in studies that evaluated eradication by endoscopy, 11.6 (95%CI: 9.9-13.3), compared to those that used non-invasive diagnostic methods, 6.6 (95%CI: 4.0-9.1).

Meta-regression

In the meta-regression, neither the study population, the method used to detect *H. pylori*, the initial antibiotic

Table 1 Characteristics of eradication trials included in Latin America

Ref.	Year	Country	Patients enrolled or randomized	Mean age \pm SD (age range)	Patient population	Treatment arm(s)	Antibiotic duration (d)	Second antibiotic treatment	Eradication success rate	Waiting time (wk)	Diagnostic method(s)	Follow-up, yr	Study design and quality ^[17]
Morgan <i>et al</i> ^[22]	2013	6 countries ^a	1463	(21-65)	Community populations	3 options: PPI + A + C PPI + A/PPI + A + M PPI + A + C + M	Variable: 14 5/5 5	PPI + M + Bis + Tetra ^b	Total 77.4% 82.20% 76.50% 73.60%	6-8	13C, CagA IgG	1	5
Silva <i>et al</i> ^[34]	2010	Brazil	150	46.7 (16-85)	Duodenal ulcer	PPI + A + C	7	PPI + Tetra + Furazolidone	92.50%	13	14C, H (RUT, PCR)	5	3
Mesquita <i>et al</i> ^[35]	2005	Brazil	50	49 \pm 14 (> 18)	Duodenal ulcer	H2 + Bis + C	14	NA	100%	13	H (RUT, H and E)	3	2
Coelho <i>et al</i> ^[36]	1991	Brazil	48	40.4 (adults)	Duodenal ulcer	A + M + Furaz	5	NA	60.40%	8.5	14C	1.5	2
Rollan <i>et al</i> ^[37]	2000	Chile	111	38 (16-75)	Duodenal ulcer	2 options: H2 + A + M PPI + A + Tinidazole	14 14	Cross-over	Total 75.7% 79% 73%	4-6	14C, H (RUT, Warthin-S, PCR)	3	3
Figueroa <i>et al</i> ^[38]	1996	Chile	57	49.1 (16-65)	Duodenal ulcer	PPI + A + M + Bis	28	NA	80.70%	4	H (RUT, Gram, Clt)	1	5
Novoa-Reyes <i>et al</i> ^[39]	2014	Peru	140	48.9 \pm 12.3 (18-85)	Non-ulcer dyspepsia	PPI + A + C	10	NA	72.10%	4	14C, H (H and E)	2	3
Soto <i>et al</i> ^[40]	2003	Peru	235	37 \pm 8.7 (18-55)	Non-ulcer dyspepsia	PPI + A + C	14	NA	85.50%	4	14C, H (Warthin-S, Clt)	1.5	5
Leal-Herrera <i>et al</i> ^[41]	2003	Mexico	467	(> 5) ^c	Non-ulcer dyspepsia	PPI + A + C	14	NA	30.20%	4-6	14C, H (Giemsa, Clt, PCR), Serology	2	4
Mohar <i>et al</i> ^[42]	2002	Mexico	131	51.4 \pm 9.3 (> 40)	Healthy volunteers	PPI + A + C	7	NA	76.30%	6	H (H and E, Elisa), CagA IgG	1	4
Sivapalingam <i>et al</i> ^[43]	2014	Bolivia	848	(> 6 mo) ^d	Community populations	PPI + A + C	10	"Triple therapy"	64.00%	6	13C, CagA IgG	1	3
Mera <i>et al</i> ^[19,44]	2005	Colombia	976	50.8 (29-69)	Intestinal metaplasia	Variable (the majority A + M + Bis)	14	NA	51.60%	156	13C, H (H and E, Steiner)	16	5

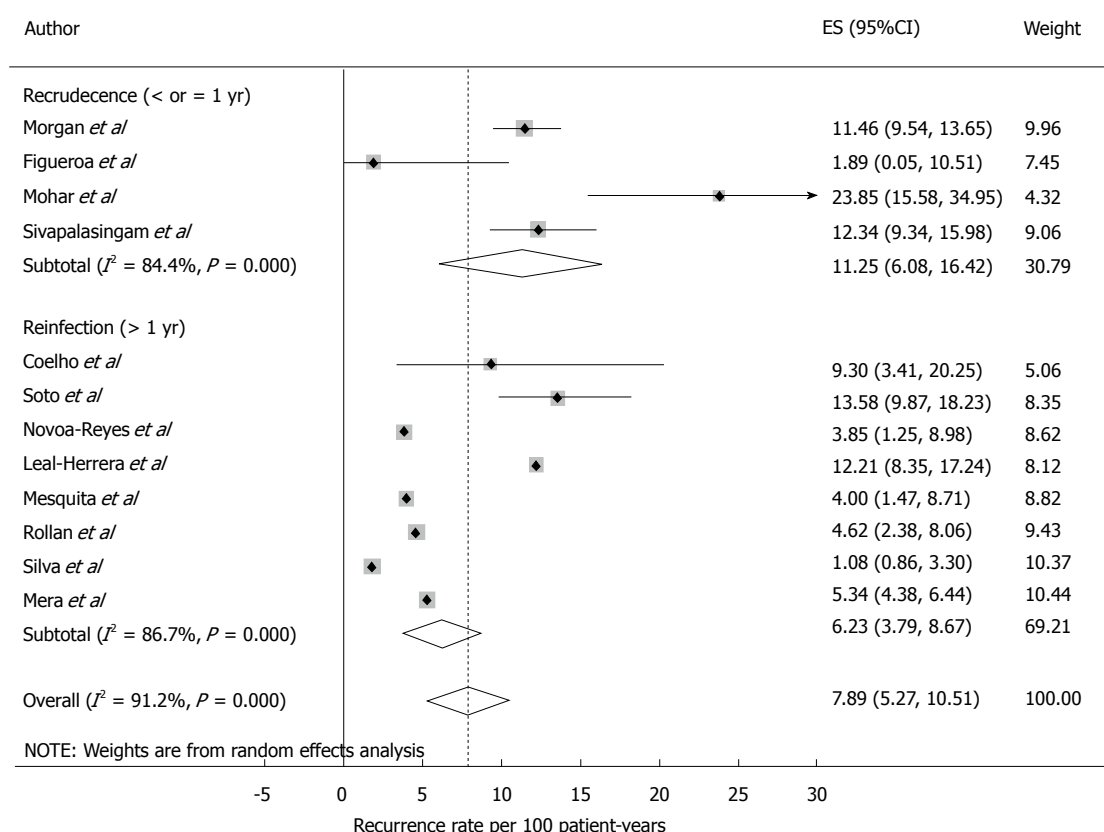
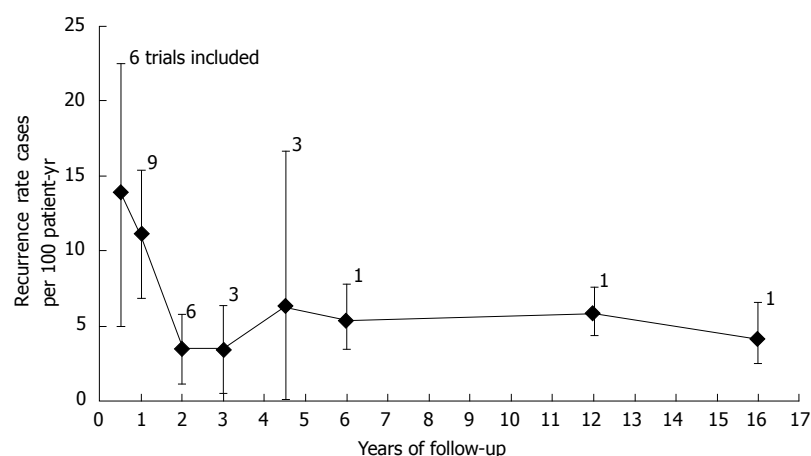
^aSix countries were Colombia, Costa Rica, Nicaragua, Chile, Honduras, and 2 sites in Mexico (Sonora and Chiapas); ^bVoluntary treatment; ^c66.7% were > 30 years old; ^d41.2% were > 15 years old. PPI: Proton pump inhibitor; A: Amoxicillin; C: Clarithromycin; H2: H2 Blockers; Bis: Bismuth; Furaz: Furazolidone; C: Urea breath test; H: Histology; RUT: Rapid urea test; Clt: Culture.

strategy, the use of a second antibiotic course, nor the length of follow up had a significant impact on the observed risk of reinfection. As anticipated, the recurrence rates decreased after the first year by 40%, but this was not statistically significant after including all five variables ($P = 0.6$).

Assessment of bias and heterogeneity

Risk of bias according to Camargo scale ranged from 2 to 5 points. Even though reviewed studies used various

methods to assess *H. pylori* recurrence, 10 (83%) used at least two different methods, including 9 (75%) that used urea breath tests. Most studies lost points because of sampling techniques or because they failed to describe salient patient characteristics. The I^2 for the model was 90% and adjusted R2 was -38.8%. Funnel plot showed asymmetry towards higher recurrence rates, with a lack of missing studies with low sample size (high SD) where the rates are lower. The top of the funnel plot demonstrated a low risk for publication bias.

Figure 2 Forest plot of *Helicobacter pylori* recurrence rates in Latin America.Figure 3 Yearly trends of *Helicobacter pylori* recurrence after eradication treatment in Latin America.

DISCUSSION

H. pylori recurrence after eradication is a critical determinant of the efficacy of potential gastric cancer prevention programs utilizing antibiotic treatment. This measure may be more important than the choice of initial antibiotic regimen and bacterial resistance rates, and is likely to differ by global region^[19-21]. Latin America populations have high colonization rates of *H. pylori*, as well as a significant burden of gastric adenocarcinoma. Our meta-analysis estimates a recurrence rate of 7.9 cases per 100 PYs in Latin America, 11.2 in year-one

and 6.2 in subsequent years. This overall rate is higher than the estimated global recurrence rate of 4.5 (95%CI: 4.2-4.8), but significantly lower than that reported for resource-limited nations 8.7 (7.8-9.6) and 13.0 (6.0-21.0) observed in two independent meta-analysis^[16,18].

Is there a maximum *H. pylori* infection recurrence threshold for potential intervention programs? In the Shangdong trial reported by Ma *et al*^[10] with healthy volunteers in East Asia, *H. pylori* eradication significantly reduced incident gastric cancer compared to placebo after 14.8 years of follow up [OR 0.6 (0.4-0.9), $P = 0.3$]. These results have been supported by recent

Table 2 Estimated *Helicobacter pylori* recurrence rates in Latin America studies

Ref.	Patients that received antibiotics	Patients present at f/u appointment	Recurrent cases total	Crude reinfection rate ¹	Follow-up (yr)	Year patients (present at f-u appointment)	Recurrence rate per 100 PY (95%CI)
Morgan <i>et al</i> ^[22]	1133	1091	125	11.46	1	1091	11.46 (9.54-13.65)
Silva <i>et al</i> ^[34]	147	112	10	8.98	5	557	1.80 (0.86-3.30)
Mesquita <i>et al</i> ^[35]	50	50	6	12.00	3	150	4.00 (1.47-8.71)
Coelho <i>et al</i> ^[36]	29	43	6	13.95	1.5	64.5	9.30 (3.41-20.25)
Rollan <i>et al</i> ^[37]	84	96	12	12.50	3	260	4.62 (2.39-8.06)
Figueroa <i>et al</i> ^[38]	47	53	1	1.89	1	53	1.89 (0.05-10.52)
Novoa-Reyes <i>et al</i> ^[39]	101	65	5	7.69	2	130	3.85 (1.25-8.98)
Soto <i>et al</i> ^[40]	201	216	44	20.37	1.5	324	13.58 (9.87-18.23)
Leal-Herrera <i>et al</i> ^[41]	141	131	32	24.43	2	262	12.21 (8.35-17.24)
Mohar <i>et al</i> ^[42]	183	109	26	23.85	1	109	23.85 (15.58-34.95)
Sivapalasingam <i>et al</i> ^[43]	543	462	57	12.34	1	462	12.34 (9.34-15.98)
Mera <i>et al</i> ^[19,44]	679	126	108	85.37	16	2024	5.34 (4.38-6.44)
Total	3338	2554	432	16.92		5487	7.89 (5.27-10.51)

¹Crude reinfection rate: Recurrent cases total/Patients present at follow-up appointment.

Table 3 Implementation of *Helicobacter pylori* eradication programs for gastric cancer prevention in Latin America

Components	Challenges and considerations	Implementation approaches
Public policy	Lack of awareness among the Ministries of Health, stakeholders, and the public	Large scale education campaigns for cancer and gastric cancer Joint initiatives with international stakeholders: WHO, IARC, PAHO, UICC, NCI, and CDC
Economic investment	Cost of <i>H. pylori</i> eradication program	Conduct CEAs at the country and regional level. The CEAs may differ for HICs and LMICs
Program design	Economics of growing gastric cancer burden Uncertainties and regional variation for target age, screening approach, treatment regimen, and follow-up	Pilot-test eradication campaigns and perform community implementation trials Adapt evidence from cost-effectiveness models and available epidemiologic data. Incorporate screening into existing public health practices (<i>e.g.</i> , cervical cancer)
Appropriate technologies	Technical difficulties in <i>H. pylori</i> testing Consistent eradication confirmation norms Management of high risk patients	Develop economic, point-of-care <i>H. pylori</i> testing Coordinate endoscopy protocols for high risk patients (<i>e.g.</i> , premalignant lesions) Implement information networks to coordinate eradication programs, health centers, and endoscopy centers
Adherence measures	Poor compliance with <i>H. pylori</i> eradication regimen, leading to treatment failure and increased infection recrudescence	Consider medication side effect profiles Pre-regimen counseling for common side effects Consider adherence measures, usual (<i>e.g.</i> , direct observed therapy), or novel (<i>e.g.</i> , cell phone contact)
<i>H. pylori</i> recurrence	Elevated reinfection rate may affect program efficacy and feasibility	Improve living conditions to reduce potential environmental sources of reinfection Consider the family or the village as the intervention target
Potential overall program risks and unknowns	Alteration of the human microbiome Induction of antibiotic resistance Potential increased risk for certain diseases (<i>e.g.</i> , allergic diseases, esophageal cancers) Unknown role(s) of <i>H. pylori</i> as a component of the human microbiome: Commensal and pathogen, which may be strain and/or age dependent	<i>H. pylori</i> eradication programs should be considered investigational, with use of rigorous methodology and long term surveillance Monitoring of antibiotic resistance and microbiome profiles Global antibiotic stewardship programs (<i>e.g.</i> , OTC antibiotic use, veterinary use)
Parallel research agendas	Incorporate evolving approaches and technologies	Develop novel biomarkers for host risk and <i>H. pylori</i> virulence Develop biomarkers for premalignant lesions (<i>e.g.</i> , intestinal metaplasia) to facilitate endoscopy surveillance Incorporate endoscopy technologies, including advanced imaging and low-cost approaches
<i>H. pylori</i> Vaccination	Unknown long-term effectiveness and side effects Lack of data showing impact in clinical outcomes	Evaluate long-term (> 3 yr) effectiveness in other centers, populations and countries ^[23] Complete regulatory evaluations, collect additional safety data and approval by national agencies. Phase IV studies

WHO: World Health Organization; IARC: International Agency for Research on Cancer; PAHO: Pan American Health Organization; UICC: Union for International Cancer Control; NCI: National Cancer Institute; CDC: Centers for Disease Control and Prevention; HIC: High income country; LMIC: Low/middle income country; OTC: Over the counter; CEAs: Cost-effectiveness analyses; *H. pylori*: *Helicobacter pylori*.

meta-analyses^[11]. In the Shangdong study, omeprazole and amoxicillin comprised both the treatment and the retreatment regimen, and only 47% of subjects were *H. pylori* negative at the 7-year post-eradication point. In general terms, this may suggest a 50% threshold at the 5 to 7 year time point as a minimum eradication efficacy target. Our estimated 5-year and 7-year reinfection rates in the current meta-analysis are lower or at least similar to the 7-year reinfection rate observed in the Shangdong study: 36.2% and 48.6%, vs 53%, respectively. Thus, *H. pylori* screening and eradication in asymptomatic populations may be an attractive strategy for gastric cancer prevention in Latin America. Further research to evaluate feasibility, cost-effectiveness, acceptance, and adverse consequences of eradication programs in the region is needed. For example, the 1-year recurrence analysis in the large 6-country *H. pylori* eradication trial in Latin America suggested that potential programs may need to be tailored based upon region, gender and age of the participants^[22]. Uncertainties about *H. pylori* screening and treatment have to be answered and significant challenges are foreseen before such programs can be implemented at a population level (Table 3).

H. pylori infection recurrence represents the combination of recrudescence and reinfection, and different strategies may be required to effectively reduce these component rates. Recrudescence or re-growth, usually occurs during the first year after treatment at a rate primarily driven by antibiotic treatment failure, in the setting of a false negative test immediately after treatment. This common scenario may be difficult to distinguish from reinfection with the same strain from a family member in the same household. Reinfection, is the principal component of recurrence after the first year, and persists at a lower but steady state. Molecular analysis comparing pre- and post-treatment strains of patients have shown that 80% of recurrent cases are genetically identical, whereas differing strains were found in only a minority of the cases^[23]. This suggests that the majority of initial recurrent cases are a product of treatment failure, or reinfection with a strain common to close contacts or family members. In this meta-analysis, recurrence rates significantly decreased after the first year and remained stable in subsequent intervals, ranging from 3.4% to 5.8% per 100 PYs.

Strategies aiming to reduce these two types of recurrence should be different. The first scenario requires a clinical approach where cost-effective antibiotic selection and medication compliance measures are crucial, whereas the second involves a broader public health strategy. Reducing reinfection rate is complex as it involves improving living conditions and reducing potential environmental sources of reinfection, including consideration of interventions at the family or the village levels, and possibly vaccination^[24]. In this approach, children become a challenging target group with higher therapeutic failure and higher reinfection as seen in most studies (including this meta-analysis)^[25-27].

Our results are significantly influenced by two trials:

The study by Morgan *et al*^[22] as the largest trial with 1463 PYs follow up, and the study by Mera *et al*^[19] as the cohort with the longest follow-up time. The Mera study was the only cohort followed for more than 5 years; subjects with preneoplastic gastric lesions were enrolled from a geographically circumscribed region of Colombia, and thus, the results may not be generalizable to the remainder of Latin America. Of note, the Caribbean was not represented, where the higher African ancestry, different diets and other environmental exposures may affect generalization. In this review, we observed geographic variability in *H. pylori* recurrence rates, as had been previously described^[22]. This likely represented both regional *H. pylori* ecology differences, as well as socioeconomic differences in the study populations. Improved socioeconomic status in subsequent birth cohorts may help explain lower acquisition rates. For example, Chile and Peru are countries with divergent development rates, yet similar ethnography and comparable *H. pylori* prevalence rates-lower reinfection rates are observed in Chile^[16,28]. One likely explanation is that in Chile, the generation ≥ 40 years who contracted *H. pylori* in their childhood and remains colonized, coexists with younger generations that have grown in improved living conditions with reduced *H. pylori* prevalence. This paradox of high prevalence but low reinfection rates has been previously described in Japanese patients with peptic ulcer disease^[29].

In our meta-regression analysis, the findings were not significantly modified by any of the evaluated factors: Study population, *H. pylori* diagnostic modality, the antibiotic strategy selected, retreatment (a second antibiotic course), or the time interval to check for *H. pylori* eradication success. Antibiotic selection varied among different studies, but half of them used the standard triple therapy regimen. This 14-d regimen has been proven to be superior to sequential and concomitant therapy in Latin America post-eradication time, but not at the 1-year time point^[22,30]. Diagnostic modalities were appropriate, and 8 out of 12 studies used two methods to diagnose *H. pylori*, wherein one of them was the urea breath test^[31]. Studies that used endoscopy-based diagnostic methods noted higher recurrence rates, which may be an incidental finding, related to occasional iatrogenic infection, or reflect the improved sensitivity of this approach^[32]. One limitation of this analysis was the study designs which were not able to differentiate whether cases were secondary to reinfection or recrudescence by molecular fingerprinting. Finally, heterogeneity was significant and there is a possibility of publication bias^[33]. The Forrest plot suggested missing studies with low sample size (wide standard deviation) wherein the recurrence rates may be lower, with the exception of the Peru study, but this is attributed to the inclusion criteria of at least 50 PYs of follow-up.

Conclusion

The meta-analysis of studies in Latin America suggests that the *H. pylori* recurrence rate in the first year is

11.2 (95%CI: 6.1-16.4) per 100 person-years, and 6.2 (95%CI: 3.8-8.7) per 100 person-years in subsequent years, or approximately 50% at 7 years. Overall, the reinfection rates are lower than initially reported, making *H. pylori* screening and eradication a reasonable strategy for gastric cancer prevention programs in Latin America, within the context of well-designed clinical trials^[18]. Further research is needed to evaluate the feasibility, cost-effectiveness, and the potential adverse outcomes (e.g., microbiome effects, antibiotic resistance) of eradication programs, while in parallel, to explore novel biomarkers and eradication strategies.

ACKNOWLEDGMENTS

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COMMENTS

Background

Recent trials and systematic reviews suggest that *Helicobacter pylori* (*H. pylori*) eradication may reduce the risk of gastric adenocarcinoma if the 5 to 7-year recurrence rate is less than 50%.

Research frontiers

There is limited evidence of *H. pylori* recurrence after treatment outside of Asia.

Innovations and breakthroughs

The authors' estimated 5-year and 7-year recurrence rates are similar to the recurrence rates observed in the literature, including the Shangdong study. Recurrent cases occur mostly within the first year suggesting treatment failure.

Applications

H. pylori screening and eradication in asymptomatic populations with chronic gastritis may be an attractive strategy for gastric cancer prevention in Latin America. Further research is needed to evaluate the feasibility, cost-effectiveness, and the potential adverse outcomes (e.g., microbiome effects, antibiotic resistance and stewardship) of eradication programs in the region.

Peer-review

This manuscript is well written and clinically interesting. Results are presented clearly and conclusions are supported by results.

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